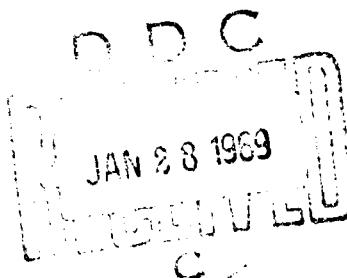


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MICROBIOLOGIC STUDIES OF THE ACTIVATED
SLUDGE PROCESS FOR THE RECYCLING
OF HUMAN WASTES

D. DUANE CHAPMAN, Ph.D.
ROBERT W. OKEY, M.S.
FREDRIC T. SANTLER, A.B.



USAF School of Aerospace Medicine
Aerospace Medical Division (AESD)
Brooks Air Force Base, Texas

September 1968

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FOREWORD

The studies on the microbiology of activated sludge described in this report were done by Scientia Research Laboratories Inc., Seattle, Wash., under task No. 793001, contract No. AF 41(609)-1974. The research was accomplished between 1 June 1963 and 31 December 1964 and was monitored by Major James E. Moyer, Environmental Systems Branch, USAF School of Aerospace Medicine. The manuscript was received for publication on 2 May 1968.

First Lieutenant Michael J. Ryan and Dr. R. L. Miner, of the Environmental Systems Branch, reviewed the report for technical accuracy.

A complete set of figures as submitted by the contractor is available in the Aeromedical Library, USAF School of Aerospace Medicine, under this title and report number. Because of the large number of illustrations submitted, it was not feasible to publish them all.

This report has been reviewed and is approved.



GEORGE E. SCHAEFER
Colonel, USAF, MC
Commander

ABSTRACT

A study was carried out to evaluate the assimilability of common urinary and fecal constituents by the active saprophytes of high-solids activated sludge. The saprophytes were obtained in pure culture from mixed cultures grown on undiluted human waste. Conventional isolation and determinative procedures were employed. The isolated organisms were found to be primarily species of *Alcaligenes*, *Pseudomonas*, and *Achromobacter*, all of which have been previously identified in activated sludge. It was observed that most organisms could assimilate a substantial quantity of the small molecules, such as uric and hippuric acids, contained in human waste. The ability to handle complex polymeric substrates, however, such as starch, cellulose, and cellulose, was found to be limited. Bilirubin, coproporphyrin, and tripalmitin were found to be refractory. The authors attribute the saprophytic activity in activated sludge to a commensal rather than to a symbiotic relationship among the organisms. Commensalism has an important bearing on synthesis of a biologic system from pure cultures. It appears that at least three organisms will be required to reproduce the activity of an activated sludge facility. Further study is recommended in these areas: more specific identification of isolated organisms; use by saprophytes of oxidation of nitrogen as an energy source; bilirubin, pyrrole, and coproporphyrin metabolism; control of synthesis in mixed cultures; and selective elimination of inactive material in the biologic matrix.

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MICROBIOLOGIC STUDIES OF THE ACTIVATED SLUDGE PROCESS FOR THE RECYCLING OF HUMAN WASTES

I. INTRODUCTION

Statement of problem

Although the activated sludge process and comparable methods employing microorganisms for the breakdown of waste materials have been in use for a number of years, very little has been determined concerning the identity of the specific organisms responsible for the major portion of the degradative processes. Although certain broad generalizations had been made, it appeared impossible to assess the role of each major group of microorganisms from the existing data.

Dependence on saprophytic organisms in a biologic waste disposal system is complete. For this reason, should the system function poorly or improperly in a remote environment because of destruction of a major class of saprophytes, the reestablishment of the system from stock cultures would be desirable. It was not known whether the general metabolic capabilities of the mixed culture could be duplicated by a combination of pure isolates obtained from the parent mixed system, or whether a selected group of such isolates could possibly improve degradative performance over that of a mixed culture derived from natural selection.

Therefore, the present work was initiated to determine the metabolic capabilities of organisms isolated from mixed microbial systems grown on undiluted human waste (the basic substrate to be received by the waste treatment system in a remote environment); then to determine if the gross assimilative capacity of the mixed system could be duplicated by a series of isolates.

Objectives

The objectives of the work were as follows:

1. Isolation and identification by standard biologic technics of microbial species present in activated sludge in the endogenous state.
2. Measurement of the rate of oxidation of components of human waste by each of the isolated organisms. These studies were to include, but not necessarily be limited to, measurement of the oxidation rate of:
 - a. Urea
 - b. Hippuric acid
 - c. Creatinine
 - d. Various sterols, taurocholate, and glycocholate
 - e. Albumin
 - f. Cellulose and other carbohydrates
 - g. Uric acid
 - h. Ethereal sulfates
 - i. Urochrome
 - j. Selected porphyrins
 - k. Lipids and fatty acids
 - l. Synthetic detergents and soaps
3. Measurement of the oxidative capacity of each of the isolated organisms on a substrate of sterile human waste.
4. Recombination of selected organisms and measurement of their activity as in 2 and 3 above, where such combination appeared productive of a more completely capable system.

5. From cultures of high-solids activated sludge, isolation, identification, and metabolic studies of organisms adapted to the degradation of cellulose, hestianic acid, bile pigments, and other normally hard-to-oxidize components of sludge reactors.

6. Consideration of metabolic and absorptive characteristics of organisms, derived from studies under 2 and 3 above, with a view to characterization of the overall process of waste degradation by microbial cultures.

In accordance with these objectives, cultures were developed on human waste at 7-day and 13-day detention periods. Organisms were obtained in pure culture from the parent mixed cultures and subjected to Warburg examination. When applicable, the isolates were examined in substrate depletion studies to determine substrate adsorptive behavior.

Attempts were made to adapt mixed cultures to cellulose, hestianic acid, bile pigments, pyrrole, and some related polycyclic substances. In those instances in which growth or metabolism appeared to occur, the organisms were obtained in pure culture.

The isolates were combined and subjected to Warburg studies to determine if the extent of substrate metabolism shown by the mixed parent culture could be accounted for.

Studies were carried out to evaluate the adsorptive characteristics of pure cultures employing substrates known to be adsorbable.

The metabolic and determinative data have been evaluated and summarized.

II. SUMMARY

A study of the metabolic characteristics of the bacteria present in activated sludge has been carried out. The organisms were isolated employing standard bacteriologic technics. After isolation, the isolates were examined in Warburg studies and in substrate depletion studies. The substrates studied were the common constituents of human feces and urine, and

additional materials such as cellulose, and soaps and detergents not easily replaced in remote environments.

From the work, the following conclusions were drawn:

1. Most of the materials contained in feces and urine are assimilated totally, or to a substantial degree, by the individual organisms present in activated sludge grown on human waste.

2. The organisms apparently responsible for the bulk of the saprophytic activity in activated sludge were found to be various species of *Pseudomonas*, *Achromobacter*, and *Alcaligenes*, and paracolon bacilli.

3. The organisms isolated from activated sludge appeared to have one of two distinct metabolic patterns. Most of the bacteria readily assimilated all, or a substantial fraction of, small individual molecules such as uric acid, hippuric acid, and creatinine. Only a few organisms, however, demonstrated a capability of assimilating large complex molecules such as albumin and starch. The organisms found to assimilate the large molecules had a markedly limited ability to utilize the small molecules.

4. The ability to utilize cellulose to some degree was found to be widespread in the microorganisms in activated sludge.

5. Bilirubin, biliverdin, and pyrrole were found to be refractory. Substantial attack on these molecules could not be demonstrated. Cyclic molecules not containing nitrogen (e.g., cyclopentane and cyclohexane) were found to be degraded. The lack of attack on the nitrogen-containing substrates may be due to the lack of specific transport mechanisms.

6. Some saprophytes appeared to utilize the oxidation of ammonia as a source of energy.

7. Hestianic acid, the brown pigment contained in the effluent from high-solids activated sludge reactors, was found to resist

biodegradation and was unable to support minimum growth of activated sludge, when other substrates had been depleted.

III. LITERATURE REVIEW

While many investigators have examined the metabolic characteristics of the microorganisms contained in activated sludge, little work has been directed specifically toward an appraisal of the fate of the constituents of human waste. The explanation for this lies in the fact that, by broad classes, most waste constituents are known to be readily assimilated by microorganisms. Metabolic data of the latter type derived from studies of microbial systems have been summarized by Umbreit (40, 41). Umbreit's summaries also contain considerable information as to the pathway of degradation of proteins, amino acids, aromatic substances, and fatty substances.

The literature pertinent to the present study is reviewed in the following paragraphs.

Urea

Although few precise data are available, there appears to be little question that a wide variety of organisms can assimilate urea. The hydrolysis of urea has been studied for many years. Significantly, urease was isolated and crystallized as early as 1926 (35).

Since urea is a toxic substance, a capability to degrade urea may be classed as a defense, or detoxification, mechanism. Based on Stephenson's (35) early review of bacterial metabolism and Gale's (17) later work, it appears that urease is produced by a wide variety of organisms. In addition to its rapid hydrolysis by bacteria, urea also is reported to serve as a nitrogen source for algae (6).

The urease reaction is:



The significance of the widespread occurrence of ureases in microbial systems is particularly important to the present study because

of the substantial quantities of urea present in human waste. Work carried out at the Boeing Company (6) indicated that urea was rapidly destroyed in mixed cultures derived from human waste.

Hippuric acid or benzoylglycine

No reports were found in which the microbial metabolism of hippuric acid was specifically studied; however, crude extracts of "hippuricase" were studied as early as 1881 (15). Ellis and Walker (10) studied the enzyme much later, and although the study added data as to hydrolytic mechanism, little information has been obtained concerning the ubiquitousness of the enzyme. In a study of the activated sludge process carried out at the Boeing Company (6), it was observed that hippuric acid was readily assimilated by mixed cultures not specifically adapted to hippurate.

Hippuric acid is the amide formed between benzoic acid and glycine and has the structure:

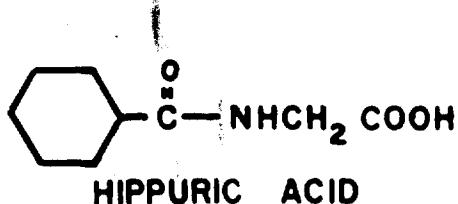


FIGURE 1

Hippuric acid is synthesized in the liver of mammals as a detoxification of benzoic acid. As a consequence, the material can be anticipated in body wastes. Bieberdorf (3) reports that average body wastes will contain 0.6 gm. of hippuric acid per 24 hours.

The study of hippuric acid metabolism is simplified if the initial or hippuricase step is assumed to take place, for no reports were found which confirmed the presence of hippuricase in bacteria. The initial steps of hydrolysis take place as follows:

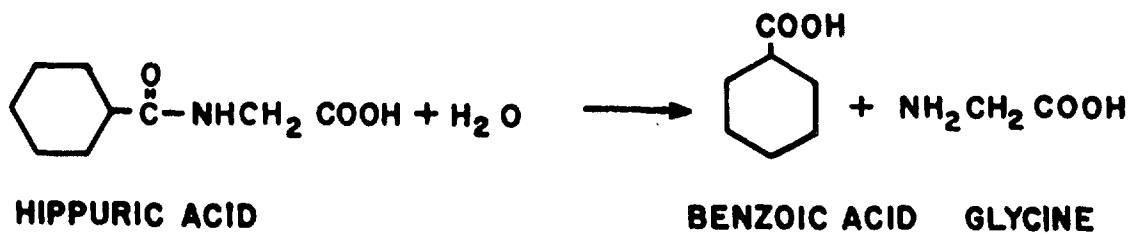


FIGURE 2

The reports of benzoic acid metabolism by mixed and pure cultures are numerous. Several are summarized in Umbreit. Okey and Bogan (26) recently published a report which included a study of benzoic acid metabolism. Symons and del Valle-Rivera (37) and Symons et al. (38) have also published detailed studies of benzoate metabolism.

Glycine metabolism has been studied with a variety of single organisms. The reports have been summarized by Fry (16). In general, glycine has been found to be rapidly metabolized. This finding was confirmed by Carlson (5), who studied the metabolism of glycine by mixed cultures similar to activated sludge. Chapman et al. (6) determined the order and rate of glycine metabolism. They found, as did Carlson, that glycine was rapidly assimilated by activated sludge organisms which had not previously acclimated to glycine.

Creatinine and creatine

In addition to the nitrogen-containing compounds previously discussed, mammalian systems discharge creatinine which arises from creatine in the tissues. Although small quantities of creatine are discharged quite normally, the bulk of the creatine destined for excretion appears as creatinine in the urine. Creatinine, therefore, is the true end product of nitrogen metabolism. The formulas for the two substances are:

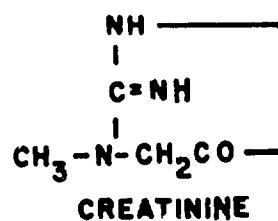
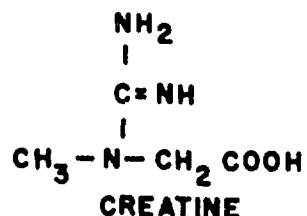


FIGURE 3

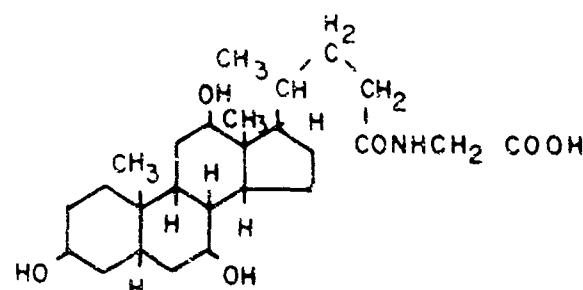
Fruton and Simmonds (15), in a summary of literature on microbial metabolism of creatine, stated that the substance was used as a nitrogen source by a number of organisms. *Pseudomonas ovalis* is reported to cleave creatine to urea and sarcosine which is then oxidized to CO_2 , H_2O , and NH_3 .

Sexton (33) reported that creatine is dehydrated to creatinine both *in vivo* and *in vitro*. Unpublished data obtained at the Boeing Company indicated that algae can use both creatine

and creatinine as nitrogen sources. These results imply that the molecule is catabolically utilized as well.

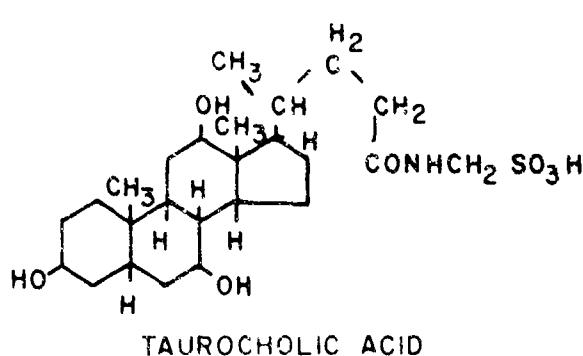
Taurocholate and glycocholate

Taurocholic and glycocholic acids are the conjugates of the bile acid, cholic acid, with the amino acids, taurine and glycine. The bile acid is bound to the amino acids through an amide linkage. The structures of glycocholic and taurocholic acids are shown below:



GLYCOCHOLIC ACID

FIGURE 4



TAUROCHOLIC ACID

FIGURE 5

Cholic acid is derived in mammalian tissue from cholesterol. The A and B rings of cholic acid are *cis* to *cis* as in coprostanol. Since no further oxidation of the bile acids takes place in mammalian tissue, these substances represent a true end product of cholesterol metabolism. Stodola (36) has summarized a substantial body of literature on the general topic of biochemical alterations of steroids. There

is, however, a distinct paucity of data dealing with the total assimilation of the general class of sterol molecules. Chapman et al. (6), in studying the metabolism of a number of human waste constituents, carried out one study on cholesterol; they found that after a short lag the cholesterol was rapidly assimilated.

Condensed aromatic hydrocarbons containing three rings have been shown by Reiff (30) and other investigators (39) to be assimilable by soil bacteria. The effect of size on assimilability is not known; however, molecules of the size of anthracene and phenanthrene are readily assimilated and used as sole carbon sources.

Albumin

Albumin is one of the nitrogen-containing compounds known as "simple protein"—indicating that it is made up of amino acids only. Albumin derived from egg white is moderately soluble in water and possesses a molecular weight of about 44,000.

It would appear that macromolecules would require some preliminary reduction in size before microbial assimilation, probably on the cell exterior. Fry (16), in his extensive review of microbial nitrogen metabolism, pointed out that a small number of exoproteases have been purified. The ability to produce exoproteases is apparently limited; Fry reports that not all organisms have this capability. The ability to produce proteolytic exoenzymes is also markedly affected by the environment, but he reports that several microorganisms can use albumin or closely related proteins (casein and peptone).

Okey et al. (28) studied albumin in their analysis of the oxidation potential as a control system for activated sludge and found it readily assimilated by activated sludge. Chapman et al. (6) observed similar results in their studies of the high solids activated sludge process. Both noted that the substrate (albumin) appeared to be rapidly removed from the medium in a first-order or concentration-dependent fashion and to be oxidatively assimilated at a slower rate.

Carbohydrates

Starch, glucose, and cellulose were studied in the present work—starch, because it is a complex carbohydrate; and cellulose, because of the limited data available on the nature of activated sludge organisms capable of assimilating this substance. Glucose was employed as a standard substrate primarily because of the wide variety of microorganisms which aerobically utilize sugars and, further, because of the extensive information now available on the degradative pathways of glucose.

The metabolism of cellulose was studied by Okey et al. (27). The paper contains an extensive literature review which will not be repeated here. In summary, there are various microorganisms capable of excreting the two enzymes, cellulase and cellobiase, necessary for the utilization of cellulose. In the referenced study, only mixed cultures were employed and no information was obtained on the metabolism of the individual organisms utilizing cellulose. It was clear, however, that, in the human waste employed as a substrate, microorganisms were present that could use cellulose—either alone or in concert.

Uric acid

Uric acid appears in man as an end product of purine breakdown and is not a principal end product of protein metabolism. The structure of this substance is shown below:

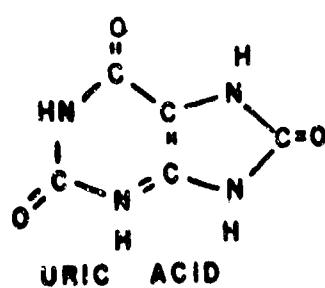


FIGURE 6

In mammals, except man and the higher apes, uric acid is oxidized and excreted as allantoin, and in fishes, the allantoin is further

oxidized to allantoic acid and then to urea and glyoxylic acid.

The purine structure is reported to be readily metabolized by a variety of both aerobic and anaerobic microorganisms. Fruton and Simmonds (15) report that *Pseudomonas* and two species of *Clostridium* utilize purine or uric acid as sole carbon and nitrogen sources.

Ethereal sulfates

Ethereal sulfates are esters of sulfuric acid, usually phenylsulfuric acid, formed in the liver of mammals. The general form of the sulfate esters is shown below:

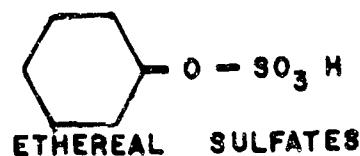


FIGURE 7

Microorganisms assimilate such esters through the hydrolysis of the esters by sulfatases, producing an organic residue, usually phenol, which can be rapidly metabolized.

Although not a urinary excretion product, alkyl sulfates (such as lauryl sulfate, a detergent substance) have also been shown to be rapidly metabolized by unacclimated, broad-spectrum, bacterial cultures such as activated sludge (6).

Urochrome

Urochrome is the major normal urinary pigment, excreted to the extent of about 73 mg. per day. Although its constitution and precursor are unknown, its output has been found to be relatively constant, independent of diet, but varying with basal metabolism. The excretion of this pigment is increased by tissue

breakdown, starvation, or by administration of acids. Very little has been successfully accomplished in its identification beyond isolation (9). Little is known, either, concerning the metabolism of urochrome by bacteria.

Porphyrin and bile pigments

Porphyrins is the general name given to a group of compounds containing the porphyrin nucleus, as shown below:

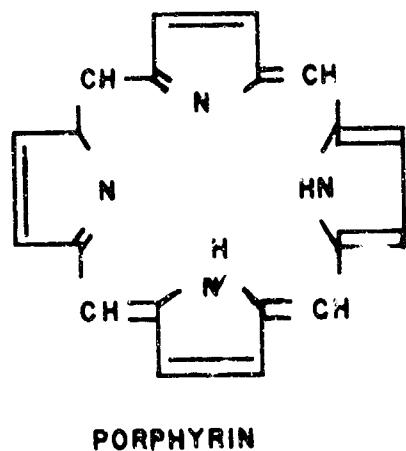


FIGURE 8

The porphyrin structure contains 4-pyrrole rings bridged by methylene groups at the carbon adjacent to the heteronitrogen. The porphyrins vary from one another by differences in extent and nature of ring substituents.

The porphyrin of importance in the study of human waste assimilation is coproporphyrin, found in human feces, the structure of which is shown in figure 9.

The porphyrins are closely related to the bile pigments. The latter substances are linear tetrapyrroles created by the oxidative degradation of porphyrin. Little is apparently known about the fate of the bile pigments. No reports of the oxidative degradation of bilirubin have been found.

The fate of coproporphyrin and the bile pigments may be related to the fate of pyrrole, the fundamental constituent molecule. Concerning the fate of pyrrole in mammalian and microbial systems, little is known. Williams (44) in his literature summary indicated that the evidence concerning the fate of pyrrole ingested by mammals is unreliable. Some, apparently a large fraction, is excreted unchanged. Some appears as urea nitrogen, while some is reported to appear, after exposure to

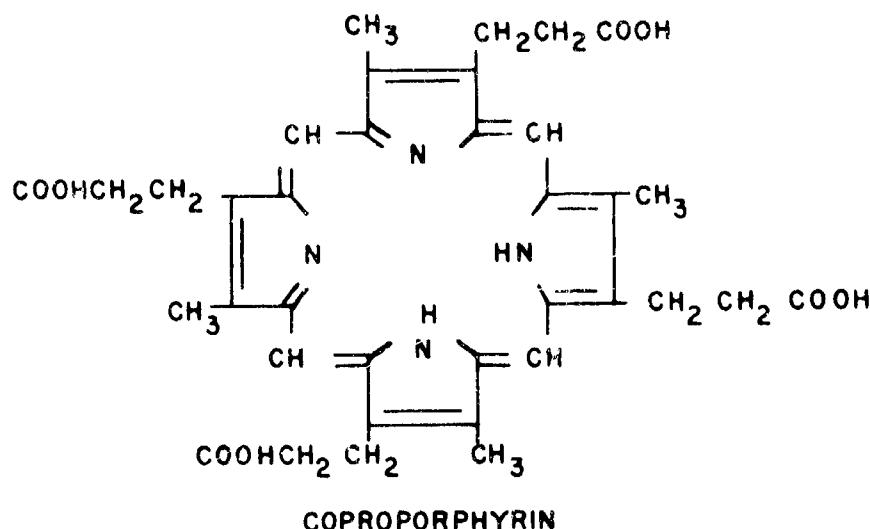


FIGURE 9

the air, as a substance similar to melanin. There is also some evidence to suggest pyrrole can give rise to pyriline in mammals (44).

Porphyrins contained in mammalian hemoglobin undergo oxidative cleavage to some linear tetrapyrroles termed bile pigments. The bile pigments, bilirubin and biliverdin, have been selected for study in this work. Although bilirubin appears to be modified by intestinal organisms, there are little data which indicate it can be completely assimilated.

Many nitrogen-containing substances are known to be toxic to fungi (18, 22). However, some heterocyclic substances are reported to be available as microbial substrates. Pyridine (12); hydroxyproline (1); uracil, and some related materials (43); nicotine (19); and pyrimidine (8) are reported to be assimilable. Aminotriazole, the nucleus of which is a five-membered ring containing 3-heteronitrogen atoms, is reported not to be assimilated by activated sludge (22).

It would appear axiomatic that some mechanisms should exist in bacteria for the assimilation of the molecular fragments that make up the tetrapyrrole in the basic heme configuration. This reasoning is based on the fact that aerobic microbial systems contain the porphyrin structure in the cytochrome systems as a part of their electron transport process. It is reasonable to assume that some mechanism must exist for the conservation of the nitrogen and energy contained in porphyrin. It may be postulated that if pyrrole alone is not assimilated, porphyrin probably is.

Lipids and fatty acids

Corn oil, castile soap, and cholesterol have been previously studied and found to be assimilated by activated sludge (6). Hence, for the purpose of this work, fatty acids of known makeup were selected for study. In addition, lipases are common in fungi.

Alkyl fatty acids, regardless of size, appear to be oxidatively attacked and degraded in

much the same fashion—that is, first by activation with coenzyme A, and thence through a series of β -oxidation steps which feed active acetate (acetyl coenzyme A) into the trichloroacetic acid cycle by a condensation reaction with oxaloacetic acid to form citric acid. Many bacterial systems are reported to carry out β -oxidation or similar processes (15, 24, 40, 41). In addition, many microorganisms use fats or fatty acids as storage products (14, 35).

Synthetic detergents and soaps

As soaps are the sodium or potassium salts of long-chain fatty acids (16- to 20-carbon atoms) the literature pertinent to the metabolism of soaps has been covered in the previous section. This review will be limited to the microbial metabolism of synthetic detergents.

Synthetic detergents, in general, are usually one of two types—anionic or nonionic. Most of the anionic substances employed are alkylbenzene sulfonates. The nonionic substances contain alkyl lipophilic groups and amides, ethers, or esters as the hydrophilic group.

The metabolism of the alkylbenzene sulfonates has been extensively studied, primarily in mixed culture. The primary factor regulating the metabolism of these compounds by an acclimated culture appears to be the branching of the side chain which occurs in most commercial products (4, 23). Therefore, the resistance is associated with a characteristic apart from that creating the detergent characteristic. In the instance of the alkylphenyl ethers and the other nonionic substances, the number and type of hydrophilic groups employed in manufacture appeared to regulate metabolism.

One of the nonionic substances was found to be partially metabolized, in an earlier work (6). This material, Triton X-100, was selected for study. The structure of this compound is shown in figure 10.

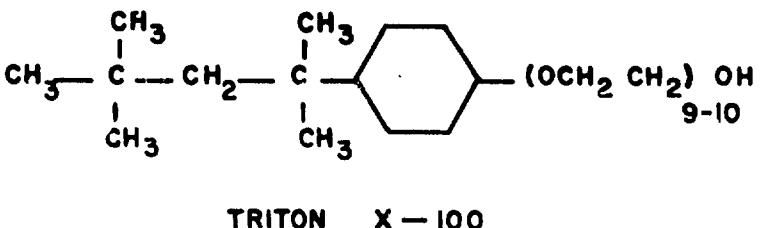


FIGURE 10

Bogan and Sawyer (4) found that Triton X-100 underwent no attack. In the Boeing study, however, substantial but probably incomplete metabolism of this compound was observed. The Boeing workers also observed that dodecyl sulfate was rapidly metabolized. The destruction of dodecyl sulfate appeared comparable to the metabolism of fatty acids. Bogan and Sawyer made a similar observation.

Despite the fact that a substantial quantity of work has been carried out on the alkylphenyl ethers, little is known of the oxidation sequence. The alkylbenzene sulfonates are oxidized from the alkyl end beginning with an α -oxidation and proceeding via β -oxidation. It is likely, however, that the ethers may be oxidized from either end, thereby accounting for the substantial oxidation despite branched-chain alkyl groups.

Microbiology of activated sludge

An extensive literature review on the microbiology of waste treatment was made by Ingram (20). Ingram's work indicated that little is known concerning the specific metabolic capability of the microorganisms found in activated sludge particularly when human waste constituents are employed as metabolites. The organisms which have been reported in activated sludge were summarized by Ingram. His list is reproduced in table I.

Large numbers of fungi and protozoa have also been found in activated sludge. Other species have been demonstrated in trickling filters. These data are summarized in table II.

TABLE I
*Microorganisms in activated sludge**

<i>Achromobacterum liquefaciens</i>
<i>Achromobacterum</i> sp.
<i>Aerobacter aerogenes</i>
<i>Alkaligenes faecalis</i>
<i>Bacillus mycoides</i>
<i>Bacillus cereus</i>
<i>Beggiatoa</i>
<i>Bacillus megatherium</i>
<i>Bacillus subtilis</i>
<i>Chromobacterium</i> (<i>Flavobacterium</i>)
<i>Crenothrix polyspora</i>
<i>Escherichia coli</i>
<i>Escherichia freundii</i>
<i>Escherichia intermedium</i>
<i>Flavobacterium lueve</i>
<i>Flavobacterium solare</i>
<i>Klebsiella pneumonia</i>
<i>Neisseria catarrhalis</i>
<i>Nitrobacteria</i>
<i>Nitrosomonos</i> sp.
<i>Nocardia actinomorpha</i>
<i>Paracolobactrum aerogenoides</i>
<i>Pseudomonas</i> sp.
<i>Pseudomonas pavonacea</i>
<i>Pseudomonas perlurida</i>
<i>Pseudomonas tralucida</i>
<i>Pseudomonas salopium</i>
<i>Sphaerotilus</i> sp.
<i>Zoogloea ramigera</i>

*As reported in reference 20.

TABLE II
Microorganisms in trickling filters*

<i>Actinomyces spp.</i>
<i>Aerobacter aerogenes</i>
<i>Alcaligenes spp.</i>
<i>Alcaligenes bookeri</i>
<i>Alcaligenes faecalis</i>
<i>Bacillus alvei</i>
<i>Bacillus cereus</i>
<i>Bacillus circulans</i>
<i>Bacillus megatherium</i>
<i>Bacillus violaceus</i>
<i>Bacillus pumilus</i>
<i>Bacillus subtilis</i>
<i>Beggiatoa sp.</i>
<i>Beggiatoa alba</i>
<i>Chromobacterium janthinum</i>
<i>Cladotrichz sp.</i>
<i>Cladotrichz dichotoma</i>
<i>Colon bacillus</i>
<i>Coli aerogenes</i>
<i>Escherichia coli</i>
<i>Escherichia coli, intermedium</i>
<i>Flavobacterium spp.</i>
<i>Flavobacterium aquatile</i>
<i>Flavobacterium balustinum</i>
<i>Flavobacterium devorans</i>
<i>Leptothrix ochracea</i>
<i>Magneta bacillus</i>
<i>Nitrobacter sp.</i>
<i>Nitrosomonas sp.</i>
<i>Nocardia spp.</i>
<i>Rhizobium ruficiculum</i>
<i>Sphaerotilus sp.</i>
<i>Sphaerotilus natans var. carnea</i>
<i>Sphaerotilus natans var. compacta</i>
<i>Sphaerotilus natans var. natans</i>
<i>Sphaerotilus natans var. uva</i>
<i>Spirillum sp.</i>
<i>Streptococcus faecalis</i>
<i>Streptomyces spp.</i>
<i>Thiotrichz nivea</i>
<i>Zoogloea filipendula</i>
<i>Zoogloea ramigera</i>

*As reported in reference 20.

The organisms listed in table III are reported by Ingram to be common to trickling filters and activated sludge.

TABLE III

Microorganisms common to activated sludge and trickling filters: bacteria and filamentous forms*

<i>Aerobacter aerogenes</i>
<i>Alcaligenes faecalis</i>
<i>Bacillus cereus</i>
<i>Bacillus megatherium</i>
<i>Bacillus subtilis</i>
<i>Beggiatoa sp.</i>
<i>Chromobacterium sp.</i>
<i>Cladotrichz</i>
<i>Escherichia coli</i>
<i>Escherichia coli, intermedium</i>
<i>Flavobacterium sp.</i>
<i>Nitrobacter</i>
<i>Nocardia sp.</i>
<i>Sphaerotilus sp.</i>
<i>Zoogloea ramigera</i>

*As reported in reference 20.

Ingram also reported that the floc present in activated sludge is responsible for the adsorption phenomena. While he presented no data to support this contention, the likelihood and significance of this hypothesis cannot be ignored. The consequence would be that the dispersed growth of young pure cultures would not adsorb, and that a treatment facility operating as a dispersed-growth unit might not adsorb waste constituents as efficiently as an older culture.

Constituents of human waste

The work reported here has been devoted primarily to an analysis of the effect upon the biodegradability of the major classes of waste constituents by high-solids activated sludge. Data as to makeup of human waste, both feces and urine, and the daily amounts produced by man are presented in tables IV and V (7). These data have been used as a guideline in selecting the substrates employed in the present work.

TABLE IV
Composition of urine

Substance (unit)	Usual range
Water	
Specific gravity (vs. water)	1.008 - 1.030
Total solids (gm./100 ml.)	1 - 7
Water (ml./100 ml.)	95 - 99
Hydrogen ion concentration (pH)	4.8 - 7.5
Minerals	
Bromine (mg./day)	1 - 5
Calcium (gm./day)	0.2 - 0.5
Chloride, as NaCl (gm./day)	10 - 15
Chromium (mg./day)	0.1 - 0.4
Copper (mg./day)	0.1 - 0.5
Fluorine (mg./day)	0.2 - 0.5
Iodine (μ g./day)	35 - 75
Iron (mg./day)	Below 1.0
Lead (mg./day)	0.05 - 0.5
Magnesium (mg./day)	1.0 - 2.0
Molybdenum (μ g./day)	10 - 30
Nickel (μ g./day)	20 - 30
Phosphorus (gm./day)	0.8 - 2.0
Potassium (mEq./day)	20 - 64
Sodium (mEq./day)	150 - 197
Solids, total (gm./day)	20 - 60
Sulfates, total (gm./day)	0.6 - 1.0
Thiocyanate (mg./day)	5 - 8
Water (liter/day)	0.5 - 1.5
Zinc (mg./day)	0.3 - 0.4
Nitrogen and protein derivatives	
Albumin (mg./day)	Less than 100
Alpha amino nitrogen, total (mg. N/day)	300 - 700
Alpha amino nitrogen, bound (mg. N/day)	100 - 200
Alpha amino nitrogen, free (mg. N/day)	120 - 240
Allantoin (mg./day)	25 - 35
Arginine (mg./day)	50 - 150
Creatine (mg./day)	Less than 100
Creatinine (gm./day)	1.0 - 1.5
Glutamine (mg. N/day)	10 - 15
Glycine (mg./day)	170 - 230
Histidine (mg./day)	180 - 250
Isoleucine (mg./day)	17 - 20
Leucine (mg./day)	20 - 30
Lysine (mg./day)	73 - 100
Methionine (mg./day)	8 - 12
Nitrogen, total (gm./day)	10 - 18
Threonine (mg./day)	50 - 65
Tryptophane (mg./day)	200 - 400
Urea (gm./day)	10 - 40
Uric acid (gm./day)	0.2 - 2.0
Valine (mg./day)	19 - 25
Xanthine (mg./day)	20 - 40

TABLE IV (contd.)

Substance (unit)	Usual range	
Carbohydrates and derivatives		
Alpha-ketoglutaric acid (mg./day)	21	- 44
Citric acid (mg./day)	210	- 470
Fructose (mg./day)	0	
Pyruvic acid (mg./day)	10	- 25
Total reducing sugar (mg./day)	0	- 100
Fats and derivatives		
Acetone bodies, total (mg./day)	10	- 100
Cholesterol (mg./day)	0.3	- 1.0
Vitamins		
Ascorbic acid (mg./day)	6	- 18
Biotin (μ g./day)	10	- 22
Choline (mg./day)	2	- 4
Niacin (mg./day)	0.2	- 1.0
N ¹ -Methylnicotinamide (mg./day)	4	- 12
N-Methyl-2-pyridine-5-carboxylamide (mg./day)	5	- 10
Pantothenic acid (mg./day)	1.0	- 3.5
Pyridoxine (mg./day)	0.05	- 0.4
Riboflavin (mg./day)	0.4	- 1.50
Thiamine (mg./day)	0.1	- 0.4
Vitamin A (IU/day)	0	
Hormones		
Androsterone (mg./day)	1	- 2
Androgen (mg./day)	3	- 10
Corticosterone (mg./day)	0.1	- 0.3
Cortin (mg./day)	0.2	- 2.5
11-Desoxycorticosteroid (mg./day)	0.1	- 0.5
Estrogens, female (μ g./day)	50	- 150
Estrogens, male (μ g./day)	22	- 26
Glycogenic steroids (mouse units/day)	40	- 80
17-Ketosteroids, female (mg./day)	5	- 14
17-Ketosteroids, male (mg./day)	8	- 20
Enzymes		
Diastase (units/day)	8	- 32
Phosphatase, acid (King-Armstrong units)	80	- 300
Trypsin (units)	40	- 250
Uropepsin (units)	1,000	- 3,000
Pigments		
Coproporphyrin, type I (μ g./day)	15	- 90
Coproporphyrin, type III (μ g./day)	1	- 24
Indican (mg./day)	40	- 150
Urobilinogen (mg./day)	0	- 4
Uroporphyrin (mg./day)	0	
Blood gases		
Carbon dioxide (mEq./day)	0	- 50
Measurements of clinical interest		
Phenols, conjugated (mg./day)	15	- 40
Phenols, free (mg./day)	0.2	- 0.4
Phenols, total (mg./day)	15	- 40

The values shown are normal ranges for various substances in human urine; all values are for healthy individuals in a resting condition at sea level in a temperate environment. The values for many substances are closely related to dietary intake and to exercise.

TABLE V
Composition of feces

Substance (unit)	Usual range
Water	
Specific gravity (vs. water)	1.030 - 1.100
Total solids (gm./100 gm.)	15 - 35
Water (ml./100 gm.)	65 - 85
Hydrogen ion concentration (pH)	7.0 - 7.5
Minerals	
Aluminum (mg./day)	1.5 - 2.9
Calcium (gm./day)	0.1 - 1.0
Chloride (mEq./day)	Traces, except in diarrhea
Copper (mg./day)	1.5 - 2.11
Iron (gm./day)	0.7 - 1.0
Lead (mg./day)	0.3 - 0.4
Manganese (mg./day)	1.9 - 2.4
Molybdenum (mg./day)	2 - 4
Nickel (mg./day)	5 - 10
Phosphorus (gm. P/day)	0.9 - 1.7
Potassium (mEq./day)	19.2 - 22.6
Sodium (mEq./day)	Traces, except in diarrhea
Tin (mg./day)	0.5 - 1.7
Zinc (mg./day)	5 - 10
Nitrogen and protein derivatives	
Arginine (gm./day)	1.2 - 2.1
Histidine (gm./day)	0.6 - 0.8
Isoleucine (gm./day)	1.4 - 2.3
Leucine (gm./day)	1.8 - 2.9
Lysine (gm./day)	1.9 - 2.9
Methionine (gm./day)	0.5 - 0.8
Nitrogen, total (gm./day)	0.7 - 2.1
Threonine (gm./day)	1.4 - 2.2
Valine (gm./day)	1.5 - 2.6
Carbohydrates and derivatives	
Total reducing sugar (mg./day)	0

TABLE V (contd.)

Substance (unit)	Usual range
Fats and derivatives	
Total fat (gm./day)	1 - 7
Total fat (percent by weight, dry)	10 - 25
Total fat, unsaponifiable (percent by weight, dry)	0 - 5
Vitamins	
Beta-carotene (mg./day)	1.7 - 3.3
Biotin (μ g./day)	100 - 200
Niacin (mg./day)	3.5 - 5.5
Pantothenic acid (mg./day)	1.8 - 3.8
Pyridoxine (mg./day)	0.1 - 0.5
4-Pyridoxic acid (mg./day)	0.5 - 0.6
Riboflavin (mg./day)	0.4 - 1.20
Thiamine (mg./day)	0.2 - 0.8
Vitamin A (mg./day)	0.17 - 0.33
Hormones	
No data available	
Enzymes	
No data available	
Pigments	
Porphyrin, total (μ g./day)	300 - 400
Protoporphyrin (μ g./day)	20 - 300
Urobilinogen (mg./day)	40 - 280
Gases	
No data available	
Measurements of clinical interest	
Bacterial debris (percent of weight, dry)	10 - 30

The values shown are normal ranges for various substances in human feces; all values are for healthy individuals in a resting condition at sea level in a temperate environment. The values for many substances are closely related to dietary intake. In addition, there are large differences from person to person.

IV. EXPERIMENTAL METHODS AND PROCEDURES

Culturing procedures

The mixed cultures from which the pure cultures studied in this work were obtained were grown on undiluted human waste. The parent mixed cultures were grown at 7-day and 13-day detention times and the organisms recovered from each system were identified and tested separately. Similar methods were used for the recovery, isolation, and identification of organisms obtained from cultures acclimated to specific substrates.

Raw waste was collected from donors on conventional diets. The feces and urine were collected separately and a standard waste mixture prepared as needed. The standard waste mixture, based on data supplied by Chapman et al. (6), consisted of the following:

Feces volume	200 ml.
Urine volume	1,800 ml.
COD ¹ (adjusted to)	25 gm./liter

The cultures grown on raw waste received only that substrate. No other inocula were intentionally added. The mixed systems were started by adding $\frac{1}{7}$ and $\frac{1}{13}$ of the culture-tube volume (300 ml.) of raw waste. Initially, during the first few days of culturing, additional waste was withheld until the culture had lost its odor of mercaptans and skatols. The waste quantity was slowly increased until each system was receiving $\frac{1}{7}$ and $\frac{1}{13}$ of its volume daily; hence the cultures had 7-day and 13-day detentions, respectively. Each day an amount of mixed liquor was removed and centrifuged. Supernatant was discarded in a volume equal to the daily volume of waste added; the cells contained in this mixed liquor were returned to the culturing reactor.

The cultures were grown at 32°C. in a constant-temperature water bath. Each system was aerated with air dispersed in the bottom of the culture through a glass tube.

¹Chemical oxygen demand.

Although foaming was a continuing nuisance, antifoams were not employed because it was feared that alterations in surface energy could affect the nature of the microbiota recovered.

The cultures adapted to bilirubin, pyrrole, hestianic acid, and cellulose were also grown in 300-ml. tubes under dispersed air at 32°C. The culture grown on, or adapted to, a single substrate received an inorganic nitrogen and phosphorus source. The inorganic adjunct employed a phosphate buffer system with ammonia as the nitrogen source to provide a COD:nitrogen ratio of 10. The latter ratio is well within the limits prescribed by Sawyer (31) in his work on activated sludge nutrition.

During the course of the work, 8 mixed cultures were developed on which some determinative bacteriology was carried out. Not all were carried to the point where species could be determined, nor were metabolic studies carried out on all the cultures. There was concern, however, regarding the general reproducibility of the response to the differential media employed. The work was done to demonstrate the reproducibility. The 8 cultures developed from raw waste for this work employed the following substrates:

1. Cellulose (developed by acclimating human waste cultures)
2. Human waste (13-day detention time)
3. Bilirubin (developed by acclimating human waste cultures)
4. Cellulose (developed by acclimating human waste cultures)
5. Human waste (7-day detention time)
6. Human waste (7-day detention time)
7. Human waste (13-day detention time)
8. Cellulose (developed by acclimating human waste cultures)

Bacterial isolation methods

The initial isolation of organisms from the 8 cultures was accomplished by aseptically

transferring a representative sample to each of the following media:

- Blood agar (Difco blood agar base B45 plus 20% citrated rabbit cells)
- Eosin methylene blue (EMB) agar (Difco B76)
- Mueller-Hinton agar (Difco B-252)
- MacConkey's agar (Difco B75)
- Tomato agar (Difco B-389)
- Salmonella-Shigella agar (Difco B74)
- Desoxycholate agar (Difco B273)
- Desoxycholate citrate agar (Difco B274)
- Dextrose broth (Difco B63)
- Nutrient broth (Difco B3)

After initial inoculation onto the medium, seeded cultures were incubated 24 hours at 37° C.; then representative colonies were taken from the various media and transferred to nutrient agar slants (Difco B1) and stock culture medium (Difco B54).

Subcultures from the 8 specimens were identified by alphabetic letters following the specimen numeral. Representative colonies were as listed:

1. 1A, 1B
2. 2A, 2B, 2FC, 2GHI, 2D
3. 3AF, 3BCDE
4. 4A, 4B, 4C, 4D, 4E, 4F, 4G, 4H, 4I
5. 5ACD, 5B, 5EFG
6. 6A, 6B, 6C, 6D
7. 7A, 7B, 7C, 7D, 7E, 7F
8. 8A, 8B, 8C, 8D, 8E

Where several letters are grouped after one number (for example, 2GHI), it indicates that this organism was recovered from different media seeded from the original inoculum. All organisms were kept on stock culture media and passaged every alternate month after purity and activity checks. The preceding stock cultures were then destroyed.

Preparation of organisms for Warburg studies

The organisms for the Warburg studies were first seeded into nutrient broth and incubated at 37° C. The growth from these cultures resulted in very high control oxygen consumption. The cells were washed in an attempt to reduce the residual concentration of medium, but contamination ran high. Next, plate cultures were used to produce greater numbers of organisms. This technic consisted of inoculating three petri plates of nutrient agar by sweep inoculation. The plates were incubated for 36 hours at 37° C. The resulting growth was suspended with a buffer solution. The cream-like suspension was transferred with Pasteur pipets into carrying tubes. This procedure gave rise to a high contamination mortality.

To cut down on the contamination and to give a higher yield, 200 ml. of tryptose blood agar base (Difco B232) was sterilized in 500-ml. bottles with screw tops and allowed to solidify. Each bottle was laid on the side, thus giving a larger surface area for seeding. The agar was inoculated from a fresh culture on nutrient agar by suspending the organisms in the "water of condensation" flooding the surface of the medium. Incubation for 24 hours at 37° C. yielded a luxuriant growth in all instances. Sterile glass beads were added to the bottles with 10 ml. of sterile buffer. Gentle rocking back and forth emulsified the organisms into a smooth creamy suspension with little or no agar particles. Transfer of the suspension to carrying tubes and addition of sufficient buffer to make a final volume of 15 ml. completed the procedure. Sterility and viability checks were then run on the cultures before releasing them for the Warburg studies. The control oxygen utilization in the Warburg was still very high. To eliminate this excessive activity or at least reduce it, a modification in the technic was adopted. The sterile medium in the 500-ml. bottle was incubated, uninoculated, for two days at 37° C. This resulted in a large volume of "water of condensation" in the bottom of the bottle; i.e., 30 to 50 ml. This broth-like "water" was removed and the medium was inoculated by purely mechanical means rather than by flooding

the surface of the agar. The final pure suspension of organisms was then incubated at 37°C. for 48 hours to allow the dense mass of organisms to utilize any nutritional carryover from the washoff procedure. This method strikingly cut down on the activity of the Warburg blanks.

Warburg experimental protocol

The utilization of the test substrates by the pure and mixed cultures was determined indirectly by measuring the amount of oxygen utilized by each culture. The amount of oxygen utilized was measured with a 14-place microrespirometer (Bronwill-Warburg type). Each substrate was tested in duplicate flasks. The flasks contained 1 ml. of the culture cell tissue, 0.2 ml. of 20% NaOH in the center well, 0.1 ml. of substrate, and an appropriate amount of monobasic and dibasic ammonium phosphate to produce a COD to nitrogen ratio of 10. A final system volume of 2.2 ml. was maintained. The Warburg determinations were carried out at $32^{\circ} \pm 0.01^{\circ}$ C. The glassware used in these studies was heated for 30 minutes at 300°F. to minimize contamination. The air inlets on the manometers were plugged with cotton to filter incoming air at the time of reset.

The results of the Warburg studies were computed as outlined by Umbreit (42) and reported as oxygen utilization in micrograms versus time. The oxygen utilized is reported as the net amount and divided by the input COD to obtain the oxidation ratios.

The substrates employed in the study were introduced into the Warburg flask in aqueous form whenever possible. If the water solubility of the substrate was low, a solvent was employed. In most cases, the solvent could be evaporated by slowly refating the flasks on a shaker table, and when less volatile solvents were used, gentle heating removed the last traces of the solvent.

The substrate concentration was determined by calculating the theoretical COD from the chemical configuration, and then measuring the COD by a modified dichromate method.

When substrates were used for more than one Warburg, the COD was repeated periodically to provide an added check on substrate concentration.

The substrates were introduced into the Warburg flask with a tuberculin syringe, and 1-ml. serologic pipets. The cells were introduced with sterile 1-ml. pipets. In each instance, pipets and syringes were used for only one substrate and cleaned immediately thereafter to prevent substrate cross-contamination.

The Warburg flasks were cleaned by the following procedure:

1. Rinse with tap water to remove residual cell tissue and substrate.
2. Soak in chloroform and scrub with a brush to dissolve the silicone grease.
3. Wash with Alconox glassware detergent and rinse in tap water.
4. Soak in hot chromic acid for 10 minutes; and rinse in tap water, and finally in distilled water.
5. Dry and disinfect in 300°F. oven for 30 minutes.

Substrate depletion protocol

The substrate depletion studies were carried out by use of cultures of small volume (30 to 60 ml.) and a shaker table for aeration. The substrate in each case was sterilized to prevent contamination of the system, and the substrate depletion was followed by determining the COD of the clarified supernatant. The systems were established at a substrate concentration of 700 mg. liter and were tested until the rate of substrate depletion was apparent.

Chemical oxygen demand

The dichromate COD test used here is similar to the modification developed by Okey (25) for reducing the system volume and

the required reflux time. In an analysis of thirty substrates, Okey found it possible to reduce the reflux time from 2 hours, as suggested by Standard Methods (34), to a period of 10 to 20 minutes—and still obtain accurate and precise results. In each instance in which the modified test was used, the efficacy of the method was checked against a solution of known oxygen demand or, when this was not possible, against the long "standard" reflux time. The details of the modified COD test are as follows:

0.500 N $K_2Cr_2O_7$	5 ml.
Concentrated H_2SO_4	15 ml.
Sample volume	10 ml.
Reflux time	20 minutes

Back-titrate excess $K_2Cr_2O_7$ with 0.25 N $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$.

Nitrite and nitrate detection

The qualitative spot tests as outlined by Feigl (13) were employed to test for the presence of nitrite and nitrate.

Urochrome preparation

Urochrome was isolated essentially by the procedure of Drabkin (9). Urine was acidified with acetic acid to pH 4 and extracted with 0.1 volume *n*-butanol. After washing, the alcoholic solution was concentrated, washed with chloroform, benzene, and ethyl ether. Crystallization was induced by further concentration and addition of absolute ethanol. Yields obtained are normally about 70 mg. per daily urine output.

Differential procedure*

Carbohydrate utilization. The individual isolates obtained from the mixed cultures were tested by the following procedures. The organisms were introduced into phenol red broth (Difco B92) plus 1% specific carbohydrate.

The carbohydrates used were galactose, maltose, saccharose, dextrose, mannitol, and lactose. The cultures were incubated at 37 C. and read at 12, 18, and 24 hours, and at 3, 5, 7, and 10 days.

Carbohydrate and H_2S utilization. Carbohydrate checks and H_2S production were run on triple sugar agar slants (Difco B265); incubation was at 37 C. Readings were taken at 4, 8, 18, 24, 48, and 72 hours.

Motility and H_2S production. Three methods were used to check the motility of the organisms. Each procedure is described in detail in the following paragraphs.

1. Hanging drop. A drop of 8- to 12-hour broth culture was placed on a cover slip and examined microscopically. A single concavity slide sealed with Vaseline was used as a well.

2. U-tube. The organisms were inoculated into 0.1% agar in a U-shaped tube plugged with cotton in both arms. The tubes were incubated at 37 C. for 24 and 48 hours. Motile organisms travel from the inoculated arm past the bottom of the U and up into the uninoculated arm of the tube.

3. Sulfite indole motility (SiM) medium. The isolates were introduced into the SiM medium by the stab method. The inoculated tubes were incubated at 37 C. for 24 and 48 hours. Motile organisms showed diffuse growth or turbidity, or both—away from the line of inoculation. H_2S production was indicated by a blackening along the line of inoculation.

Indole production. SiM medium (solid) and methyl red-Voges-Proskauer medium (Difco B16 fluid) were incubated for 24 and 48 hours after inoculation. Indole production was demonstrated by (a) Kovacs's reagent and (b) Ehrlich's test.

MR-VP medium. Two tubes were inoculated and incubated at 37 C. After 12 hours, a 5-ml. sample was withdrawn from one tube and tested. The remainder of the

culture was then incubated for an additional 12 hours and examined. Werkman's test for Vöges-Proskauer reaction was used in each case.

The second tube was incubated for 5 days and the methyl red reaction carried out.

Ammonia production or urea utilization. Urea broth concentrate (Difco B280) and urea agar concentration (Difco B284) were prepared and the appropriate base sterilized in screw-top glass tubes, aseptically mixed, and inoculated. Incubation was at 37° C. and readings were taken at 24 hours and 36 hours.

Milk. Ulrich milk (Difco B251) was used instead of the more popular litmus milk as it was felt that a wider range of reactions was available with this medium, especially in the alkali-producing category of organisms. The medium dispensed in screw-top glass tubes was incubated up to 21 days at 37° C. before final reading were taken.

Milk-litmus. Litmus milk was used at the termination of the identification program in an attempt to supplement the data for more definite classification.

Gelatin. Nutrient gelatin (Difco B11) was dispensed in screw-top glass tubes and in 50-ml. glass bottles. The tubes were incubated at room temperature, whereas the bottles were incubated at 37° C. after inoculation. Stab inoculation was used in both cases. The bottled media naturally liquefied at 37° C. and after 7 days' incubation were transferred to the refrigerator until the control bottle gelled. The various cultures were then checked for the presence of liquefaction. Cultures were then reincubated for 7 days and the process of chilling and reincubation continued for a period of 50 days. Results were comparable with those of the tubes incubated at room temperature, which gave a scanty growth, but demonstrated the type of growth away from the line of inoculation.

Nitrate reduction. Nitrate agar (Difco B106), dispensed in screw-top tubes, was inoculated and incubated for 72 hours at 37° C.

The presence of nitrites, detected by sulfanilic acid and naphthylarnine reagents, indicated the reduction of the nitrates in the original media.

Sellers's differential medium. Difco 0895, a comparatively new medium, was used to confirm the absence of the *Mima polymorpha* group of organisms and to assist in the possible identification of any of the various species of *Pseudomonas* or *Alcaligenes*. Deep stab and streak inoculation of the medium followed by incubation at 37° C. for 24 hours did not demonstrate any of the *Mima polymorpha* group. All of the organisms under study were not run through this medium.

Nitrogen production. Sellers's differential medium (Difco 0895) was used as one method to demonstrate the production of nitrogen.

V. PRESENTATION OF RESULTS

In this section we discuss first the isolation and testing of the individual organisms derived from the various mixed cultures; second, the metabolism of the mixed and pure cultures, and third, the adsorption and special metabolic characteristics of the pure and mixed cultures.

Presumptive isolate identification

The bulk of the metabolism studies were carried out with organisms derived from the first 7-day and 13-day detention cultures established for this study. These were the organisms labeled 2 and 5. Considerable effort was directed toward the identification of the species of these organisms.

In addition to the work on the principal microorganisms, determinative work was carried out on the bilirubin and cellulose cultures. The results of these studies are also reported here.

Based on the results of the differential examination, which are shown in table VI, the

TABLE VI
Results of differential tests

Organism	TSI* Slant/Butt	Motility	Iodoine	H ₂ S	VP	MR	Urea slant	Urea broth	Turbid	Granular	Mucoid	Pellitic	Urea drop ^b						
2B	A	A	A	N	P	N	P	P	P	N	P	P	P	P	P	P	P	P	P
8D	A	A	A	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
2FC	N	B	N	NC/NC	P	N	P	N	N	N	N	N	N	N	N	N	N	N	N
2GHI	N	B	N	NC/NC	P	N	P	N	N	N	N	N	N	N	N	N	N	N	N
6A	N	N	B	NC/NC	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
7E	N	N	B	NC/B	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
5B	N	B	N	NC/NC	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
7D	N	B	N	NC/NC	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
6D	N	B	N	NC/NC	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
7A	N	B	N	NC/NC	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
3BCDE	N	N	N	NC/NC	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
6C	N	N	N	NC/NC	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
8E	N	N	N	NC/NC	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
6B	N	B	N	NC/NC	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
7B	N	B	N	NC/NC	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
7F	N	B	N	NC/NC	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
7C	N	B	N	NC/NC	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
8B	N	B	N	NC/NC	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
3AF	N	N	N	NC/NC	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
8A	N	N	N	NC/NC	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
2D	N	N	N	NC/NC	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
2A	N	N	N	NC/NC	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
5ACD	N	N	N	NC/NC	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
5EFG	N	N	N	NC/NC	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
8C	N	N	N	NC/NC	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N

Code:
N = Negative.
P = Positive.
A = Acid.
B = Basic (alkaline).
R = Reduced.

*Triple sugar iron agar.

BNC = Alkaline no clot.
NC = No change.
R = Reduced.

pure cultures have been tentatively identified as follows:

2GHI	<i>Pseudomonas</i>
2FC	<i>Pseudomonas</i>
2B	Paracolon
2D	<i>Alcaligenes</i> or <i>Achromobacter</i>
3AF	<i>Alcaligenes</i>
3BCDE	<i>Alcaligenes</i> or <i>Achromobacter</i>
4B	<i>Gaffkya tetragena</i>
5ACD	<i>Alcaligenes</i>
5EFG	<i>Achromobacter</i>
5B	<i>Achromobacter</i> or <i>Alcaligenes</i>
8A	<i>Bacillus sphaericus</i>
8B	<i>Nocardia</i>
8D	<i>Micrococcus ureae</i>

The colonial morphology and other pertinent data are presented in the following paragraphs:

2A Low, convex, circular; up to 1 mm. in diameter with entire edge. Grayish, glistening, gram-negative rod; slightly striated; 1.9 μ g.

2B Umbonate, circular; 2 mm. in diameter with entire edge. Creamy-white, dull, gram-negative coccobacilli (diplobacilli); 1.9 μ g.

2D Low, convex, circular; up to 1 mm. in diameter with entire face. Grayish, glistening, gram-negative rod; slightly striated; 1.9 μ g.

2FC Convex, circular; 2 mm. in diameter with entire edge. Creamy-white, dull, gram-negative diplobacilli; 0.7 μ g.

2GHI Convex with papillate surface, circular; 1 to 2 mm. in diameter with radically striated periphery and a lobate edge. Grayish, dull, gram-negative rod; 1.5 μ g.

3AF Small, gram-negative bacilli; 0.5 to 1.0 μ g.

5ACD Low, convex, circular; 1 to 2 mm. in diameter, with crenated edges. Grayish-white, dull, gram-negative, filamentous rod; 2.5 μ g.

5B Convex, circular, $\frac{1}{4}$ to $\frac{1}{2}$ mm. in diameter with entire edge. Grayish, dull, gram-negative rod; 1.5 μ g.

5EFG Low, convex, circular, 1 mm. in diameter with crenated edges. Grayish-white, dull, gram-negative rod; 2.5 μ g.

The aerobiosis study results were all positive. The results of the litmus milk study were all negative—all, no change. The results of the Seller's differential test for the following cultures were positive: 2A, 2B, 2FC, 2GHI, 6A, 6B, 6D, 7C, and 7D.

Metabolism of fecal and urinary constituents

Mixed-culture metabolism studies. Mixed cultures were grown on undiluted human waste at 7-day and 13-day detentions as previously described. The mixed cultures were subjected to a series of Warburg studies to provide a base for the metabolic studies of the pure cultures. The substrates employed were:

1. Sterile human waste
2. Urea
3. Hippuric acid
4. Uric acid
5. Taurocholate
6. Glycocholate
7. Albumin
8. Starch
9. Cellulose
10. Coproporphyrin
11. Urochrome
12. Bilirubin
13. Biliverdin
14. Creatine
15. Creatinine
16. Dextrose

Although not normal human waste constituents, the following substrates were also studied:

1. Pyrrole
2. Pyrazole
3. Cyclopentane
4. Cyclohexane
5. Hexanoic acid
6. Tripalmitin
7. Triton X-100
8. Sodium stearate
9. Phenyl sulfate

The results of the Warburg studies with the 7-day and 13-day detention cultures are presented in table VII. In evaluating the results, several correlating mechanisms were attempted. It was decided to relate the amount of oxygen used by the substrate flasks (minus the control) to the COD of the substrate added. According to available data a ratio, here termed the oxidation ratio, of approximately 0.6 represents substantially complete utilization of the substrate, the remainder of the substrate being utilized for synthesis. Unacclimated microbial systems may use a greater

fraction of the substrate for energy, presumably to synthesize the enzymes required for rapid substrate utilization.

Pure-culture metabolism studies. The series of substrates employed in the metabolism studies of mixed cultures was employed as well in the study of pure-culture metabolism.

The oxidation ratios for each substrate and organism are presented in table VIII. All metabolic data are summarized in table IX.

TABLE VII
*Oxygen uptake and oxidation ratios for mixed and pure cultures:
mixed cultures*

Substrate	Input COD ($\mu\text{g.}$)	Net O_2 utilized ($\mu\text{g.}$)	Net O_2 utilized COD input
Culture (7-day detention)			
Sulfate	990	0	No metabolism
Cellulose	1,138	0	No metabolism
Triton X-100	920	0	No metabolism
Coproporphyrin	1,890	0	No metabolism
Urochrome	7,500	20	0.00267
Raw waste	500	570	1.14
Taurocholate	392	480	1.22
Glycocholate	302	570	1.89
Creatine	294	340	1.16
Dextrose	428	520	1.21
Culture (13-day detention)			
Raw waste (1)	515	260	0.505
Raw waste (2)	990	800	0.81
Hexanoic acid	621	170	0.274
Hippuric acid	815	527	0.647
Uric acid	333	398	1.195
Starch	593	150	0.255
Coproporphyrin	803	380	0.473
Tripalmitin	862	290	0.336
Glycocholate	755	820	1.08
Taurocholate	974	660	0.68

TABLE VIII
*Oxygen uptake and oxidation ratios for mixed and pure cultures:
 pure and bulk cultures*

Substrate	Input COD ($\mu\text{g.}$)	Net O_2 utilized ($\mu\text{g.}$)	Net O_2 utilized
			COD input
2A			
Taurocholate	196	20	0.102
Glycocholate	151	40	0.265
Dextrose (1)	214	130	0.609
Dextrose (2)	460	465	1.01
Dextrose (3)	1,066	355	0.333
Creatinine	992	255	0.257
Stearic acid	730	160	0.219
Albumin	250	0	No metabolism
Raw waste (1)	530	130	0.245
Raw waste (2)	974	230	0.236
Triton X-100	920	350	0.380
Urochrome	7,500	200	0.0267
Coproporphyrin	1,890	60	0.0318
Tripalmitin (1)	335	25	0.075
Tripalmitin (2)		60	
Creatine	147	30	0.204
Sulfate (1)	500	205	0.410
Sulfate (2)	800	260	0.325
Cellulose (1)	1,138	50	0.0439
Cellulose (2)	455	110	0.242
Cellulose (3)	1,138	320	0.280
Hexanoic acid	815	0	No metabolism
Hippuric acid	750	155	0.207
Urie acid	250	170	0.680
Starch	620	0	No metabolism
2B			
Raw waste (1)	493	160	0.324
Raw waste (2)	530	170	0.321
Raw waste (3)	500	240	0.480
Dextrose (1)	533	260	0.487
Dextrose (2)	460	420	0.914
Dextrose (3)	460	40	0.087
Creatinine (1)	496	0	No metabolism
Creatinine (2)	314	0	No metabolism
Stearic acid (1)	1,460	700	0.479
Stearic acid (2)	292	105	0.360
Taurocholate	196	5	0.0255
Glycocholate	151	0	No metabolism
Coproporphyrin (1)	380	0	No metabolism
Coproporphyrin (2)	1,890	30	0.016
Creatine (1)	147	0	No metabolism
Creatine (2)	366	0	No metabolism
Sulfate	990	410	0.414
Triton X-100	920	430	0.458
Urochrome	7,500	530	0.071
Cellulose	1,138	85	0.075
Tripalmitin	335	50	0.149
Starch (1)	620	10	0.016

TABLE VIII (contd.)

Substrate	Input COD ($\mu\text{g.}$)	Net O_2 utilized ($\mu\text{g.}$)	Net O_2 utilized
			COD input
Starch (2)	386	35	0.091
Uric acid	250	235	0.940
Hexanoic acid	815	110	0.135
Hippuric acid	750	65	0.086
2D			
Tripalmitin (1)	600	25	0.0416
Tripalmitin (2)	670	0	No metabolism
Coproporphyrin (1)	756	10	0.0132
Coproporphyrin (2)	1,512	0	No metabolism
Taurocholate (1)	196	5	0.0255
Taurocholate (2)	196	55	0.280
Glycocholate (1)	151	50	0.331
Glycocholate (2)	151	40	0.265
Raw waste (1)	500	325	0.650
Raw waste (2)	252	650	0.258
Raw waste (3)	974	200	0.206
Raw waste (4)	500	590	1.18
Raw waste (5)	530	100	0.189
Dextrose (1)	214	150	0.701
Dextrose (2)	1,066	410	0.384
Creatinine	992	200	0.202
Creatine	147	120	0.816
Stearic acid	730	135	0.185
Albumin	250	0	No metabolism
Urochrome	7,500	250	0.3333
Cellulose	1,138	330	0.318
Hexanoic acid	815	365	0.448
Hippuric acid	750	125	0.168
Uric acid	250	110	0.440
Starch	620	0	No metabolism
Sulfate	990	340	0.344
Triton X-100	920	160	0.174
2FC			
Sulfate	990	50	0.050
Triton X-100	920	80	0.087
Raw waste (1)	1,030	265	0.258
Raw waste (2)	500	500	1.00
Raw waste (3)	974	220	0.226
Dextrose	1,066	400	0.376
Creatinine (1)	992	535	0.540
Creatinine (2)	199	0	No metabolism
Stearic acid	730	275	0.377
Albumin	250	0	No metabolism
Hexanoic acid	1,242	505	0.406
Hippuric acid	1,630	670	0.411
Uric acid	667	152	0.228
Starch	1,185	0	No metabolism
Urochrome	7,500	540	0.072
Coproporphyrin (1)	1,890	0	No metabolism
Coproporphyrin (2)	380	110	0.290
Taurocholate	196	148	0.755

TABLE VIII (contd.)

Substrate	Input COD ($\mu\text{g.}$)	Net O_2 utilized ($\mu\text{g.}$)	Net O_2 utilized
			COD input
Glycocholate (1)	151	10	0.066
Glycocholate (2)	302	180	0.596
Creatine	147	79	0.537
Tripalmitin (1)		0	No metabolism
Tripalmitin (2)	268	0	No metabolism
Cellulose	1,138	0	No metabolism
2GHI			
Raw waste (1)	974	190	0.195
Raw waste (2)	450	560	1.26
Raw waste (3)	515	260	0.505
Raw waste (4)	500	500	1.00
Dextrose (1)	1,066	385	0.361
Dextrose (2)	214	115	0.537
Creatinine	992	20	0.021
Stearic acid (1)	730	300	0.411
Stearic acid (2)	292	460	1.57
Albumin	250	0	No metabolism
Sulfate	990	275	0.278
Triton X-100	920	340	0.370
Cellulose (1)	1,138	210	0.184
Cellulose (2)	1,138	275	0.242
Tripalmitin (1)	268	110	0.410
Tripalmitin (2)	268	0	No metabolism
Coproporphyrin (1)	756	225	0.298
Coproporphyrin (2)	756	90	0.119
Taurocholate	196	125	0.639
Glycocholate	151	0	No metabolism
Creatine	147	120	0.816
Hexanoic acid	621	280	0.451
Hippuric acid	815	465	0.570
Uric acid	333	80	0.241
Starch	503	0	No metabolism
Urochrome	1,500	0	No metabolism
2-Bulk			
Raw waste (1)	500	930	1.86
Raw waste (2)	500	900	1.80
Cellulose (1)	1,138	300	0.264
Cellulose (2)	1,138	250	0.220
Starch (1)	482	150	0.311
Starch (2)	482	150	0.311
Creatine (1)	366	215	0.587
Creatine (2)	366	206	0.545
Creatinine (1)	392	200	0.510
Creatinine (2)	392	100	0.255
Coproporphyrin	1,880	0	No metabolism
Urochrome	7,500	0	No metabolism
Tripalmitin	335	0	No metabolism
Sulfate	990	150	0.151
Albumin	425	115	0.271
Taurocholate	196	45	0.229

TABLE VIII (contd.)

Substrate	Input COD ($\mu\text{g.}$)	Net O_2 utilized ($\mu\text{g.}$)	Net O_2 utilized
			COD input
Glycocholate	151	70	0.464
Stearic acid	292	190	0.650
Dextrose	214	115	0.536
Triton X-100	920	290	0.315
5ACD			
Sulfate	990	0	No metabolism
Triton X-100	920	0	No metabolism
Creatinine (1)	314	75	0.239
Creatinine (2)	992	610	0.615
Creatinine (3)	314	180	0.573
Cellulose	1,138	88	0.077
Urochrome	7,500	190	0.025
Tripalmitin (1)	268	2	0.075
Tripalmitin (2)	268	0	No metabolism
Raw waste (1)	500	395	0.790
Raw waste (2)	530	125	0.236
Raw waste (3)	974	350	0.359
Raw waste (4)	500	475	0.950
Hexanoic acid	815	280	0.343
Hippuric acid	750	550	0.734
Uric acid	250	150	0.600
Starch	620	50	0.081
Dextrose (1)	1,066	425	0.399
Dextrose (2)	460	170	0.369
Stearic acid (1)	730	380	0.520
Stearic acid (2)	292	85	0.291
Albumin	250	0	No metabolism
Creatine (1)	366	225	0.616
Creatine (2)	147	86	0.585
Taurocholate	196	80	0.407
Glycocholate	151	95	0.629
Coproporphyrin	380	40	0.105
5B			
Taurocholate	196	135	0.690
Glycocholate	151	195	1.29
Stearic acid (1)	292	225	0.771
Stearic acid (2)	730	0	No metabolism
Albumin (1)	560	240	0.429
Albumin (2)	500	1,675	3.35
Raw waste (1)	974	1,150	1.18
Raw waste (2)	530	240	0.452
Dextrose (1)	1,066	0	No metabolism
Dextrose (2)	460	380	0.835
Creatinine	992	0	No metabolism
Sulfate	990	90	0.091
Triton X-100	920	0	No metabolism
Tripalmitin	335	0	No metabolism
Urochrome	7,500	0	No metabolism
Coproporphyrin	1,890	50	0.026
Cellulose	1,138	180	0.158

TABLE VIII (contd.)

Substrate	Input COD ($\mu\text{g.}$)	Net O_2 utilized ($\mu\text{g.}$)	Net O_2 utilized
			COD input
Hexanoic acid	815	300	0.368
Hippuric acid	750	125	0.166
Uric acid	250	100	0.400
Starch	620	0	No metabolism

5EFG

Sulfate	990	0	No metabolism
Triton X-100	920	50	0.056
Raw waste (1)	536	135	0.255
Raw waste (2)	530	1,350	2.55
Raw waste (3)	500	455	0.910
Hexanoic acid	815	0	No metabolism
Hippuric acid	750	300	0.400
Uric acid	250	115	0.45
Starch	620	0	No metabolism
Dextrose	1,066	600	0.563
Creatine	147	95	0.674
Creatinine (1)	992	160	0.161
Creatinine (2)	992	65	0.655
Stearic acid (1)	730	325	0.445
Stearic acid (2)	292	58	0.199
Albumin	250	0	No metabolism
Coproporphyrin	1,512	85	0.0113
Urochrome	7,500	0	No metabolism
Tripalmitin	268	45	0.168
Cellulose	1,138	225	0.197
Taurocholate	196	150	0.765
Glycocholate	151	50	0.331

5-Bulk

Coproporphyrin (1)	756	155	0.205
Coproporphyrin (2)	380	145	0.382
Creatine (1)	147	220	1.49
Creatine (2)	147	215	1.46
Urochrome	7,500	385	0.051
Tripalmitin	340	0	No metabolism
Cellulose	1,183	530	0.448
Sulfate (1)	990	500	0.505
Sulfate (2)	990	210	0.212
Starch	433	500	1.105
Triton X-100	920	775	0.842
Albumin	425	380	0.895
Taurocholate (1)	196	190	0.970
Taurocholate (2)	196	235	1.20
Glycocholate (1)	151	225	1.49
Glycocholate (2)	151	315	2.09
Stearic acid (1)	292	170	0.582
Stearic acid (2)	292	170	0.582
Dextrose (1)	214	305	1.41
Dextrose (2)	214	300	1.40
Dextrose (3)	214	65	0.304
Raw waste	252	155	0.615

TABLE IX
Extent of substrate oxidation (summary)

Substrate	Organism											
	2A	2B	2D	2FC	2GHI	2-Bulk	5ACD	5EFG	5B	5-Bulk	7-Day	13-Day
Raw waste	0.245	0.321	0.189	1.00	1.26*	1.80	0.236	0.255	0.452	0.615	0.283	0.81*
	0.238	0.324	1.18*	0.258	1.00	1.86	0.790	0.910	1.18*		0.595	0.505
	0.480		0.266	0.226	0.505		0.359				0.314	
			0.650		0.195		0.950				0.145	
Dextrose	0.333	0.087	0.384	0.371	0.361	0.536	0.369	0.563	0.10	0.304	0.561	0.405
	1.01	9.914	0.701		0.537		0.399		0.835	1.41	0.094	
	6.689	0.487								1.40	0.123	1.21
Coproporphyrin	0.032	0.016	0.013	0.00	0.298	0.00	0.105	0.011	0.026	0.382	0.00	0.473
Urochrome	0.027	0.071	0.03	0.072	0.00	0.00	0.025	0.00	0.00	0.051	0.003	0.087
Triparmitin	0.075	0.149	0.00	0.00	0.410	0.00	0.00	0.168	0.00	0.00		0.336
Albumin	0.60		0.00	0.00	0.00	0.271	0.00	0.00	3.35*	0.895	0.940*	1.08*
Taurocholate	0.102	0.026	0.280	0.755	0.639	0.229	0.407	0.765	0.690	1.20	1.22	0.68
Glycocholate	0.265	0.00	0.265	0.066	0.00	0.464	0.629	0.331	1.29	2.09	1.89	1.08
Stearic acid (sodium salt)	0.219	0.360	0.185	0.377	0.411	0.650	0.291	0.199	0.771	0.582	0.752	
Triton X-100	0.380	0.458	0.174	0.087	0.370	0.315	0.60	0.056	0.00	0.842	0.00	0.071
Phenyl sulfate	0.325	0.414	0.344	0.050	0.278	0.151	0.00	0.00	0.091	0.212	0.00	
Cellulose	0.242	0.075	0.318	0.00	0.184	0.226	0.077	0.197	0.158	0.448	0.00	0.026
Starch	0.00	0.091	0.00	0.00	0.00	0.311	0.081	0.00	0.00	1.105	0.329	0.253
Creatine	0.00	0.00	0.816	0.537	0.816	0.545	0.585	0.674		1.46	1.16	
Creatinine	0.257	0.00	0.202	0.00	0.021	0.255	0.573	0.655	0.09		1.00	
Exanato acid	0.00	0.135	0.148	0.406	0.451		0.343	0.00	0.368		0.671	0.274
Hippuric acid	0.207	0.086	0.168	0.411	0.570		0.784	0.400	0.166		0.484	0.647
Uric acid	0.680	0.349	0.440	0.228	0.21		0.690	0.46	0.400		1.184	1.195

*Nitrite detected qualitatively in these studies.

Adsorptive and special metabolic studies

Substrate adsorption by pure cultures. Studies were carried out to determine the order of substrate removal by pure cultures. The cultures used in this work were selected for their ability to assimilate those substrates shown to be adsorbed in previous work. Chapman et al. (6) demonstrated that human waste components and albumin were rapidly adsorbed onto activated sludge. The removal pattern was shown to be a first-order or concentration-dependent phenomenon. The substrate depletion experiments were carried out as outlined in the experimental protocol.

The adsorption studies indicated that little or no adsorption onto the pure cultures took place.

Special metabolism studies

1. Adaption to hestianic acid. Previous work (6) has indicated that cultures of high-solids activated sludge accumulate varying quantities of a refractory brown pigment. These materials were given the general name of hestianic acid. Emanuel (11), in a detailed study of the complex mixture of materials so described, tentatively concluded that the materials were melanin-like. Although not well documented, it appeared that these substances were in a higher concentration in long detention (10- to 20-day) systems than in those operating on shorter detention times.

To evaluate the assimilability of hestianic acid, a 300-ml., 13-day detention culture was established as previously described and permitted to accumulate nestianic acid. The latter material was precipitated from the discarded supernatant each day by use of the separation procedure of Chapman (6). The precipitate was then washed, neutralized, and returned to the culture. After 30 days of such operation, the culture had accumulated 207 mg. dry weight of material which responded to the separation procedure. The hestianate was returned to the culture and the system was aerated for 30 days. At the end of the 30-day period, the separation was repeated and

200 mg. of hestianic acid were recovered. The culture was unable to assimilate glucose at this point and gave every indication of not being viable. Hence, no organisms were recovered, and no further efforts were made to adapt organisms to hestianate.

2. Adaption to bile pigments. Early work on this contract indicated that neither bilirubin nor biliverdin was rapidly assimilated by activated sludge. To examine the problem further, a culture (3-day detention) receiving only waste was fed a waste mixture enriched with 10 mg. liter of sodium bilirubinate. This mixture was fed for a period of 3 months at the end of which time the cells were subjected to Warburg study.

Sodium bilirubinate was prepared by adjusting a suspension of bilirubin to pH 7.3 with NaOH. The bilirubin culture utilized about 100 μ g. of oxygen over the blank. This constitutes about 14% to 16% of the theoretic amount based on data of Servizi and Bogan (32). This roughly correlates with the oxygen required to oxidize the side chains to carboxyl groups adjacent to the pyrrole rings. This extent of oxidation encouraged additional work in two areas—the isolation and study of single organisms from the culture exposed to bilirubin, and a specific analysis of the biodegradability of pyrrole and a group of similar compounds.

Two organisms were isolated from the culture exposed to bilirubinate—3AF and 3BCDE. The organisms were studied further in Warburg analysis. The organisms showed about the same ability to assimilate bilirubin. Biliverdin, however, appeared more refractory than bilirubin, and neither organism could assimilate pyrrole, although 3BCDE appeared to assimilate a small quantity of pyrazole. Both organisms demonstrated a substantial capability to assimilate raw waste.

The culture which was fed pyrrole as a sole carbon source was started at 10 mg. liter and increased uniformly for a week until the substrate concentration was 200 mg./liter. At this point, the cells were employed in a Warburg study.

The culture metabolized no pyrrole although the concentration in the Warburg flask was substantially the same (150 mg. liter) as the final concentration of that material fed to the culture. Interestingly, the culture exposed to pyrrole was able to metabolize substantial quantities of cyclohexanol and cyclopentane; however, no bilirubin was utilized.

The culture was reinoculated with 300 mg. liter per day of raw waste and fed both pyrrole and raw waste at that rate for 2 weeks. The cells derived therefrom were employed at that time for further Warburg studies. These data are presented in tables X and XI.

In the study employing the cells from the culture receiving both pyrrole and raw waste,

12% to 16% of theoretic gas uptake for bilirubin was again observed. In addition, some small quantity of pyrrole appeared to be utilized.

3. Adaption to cellulose. The procedure employed in the study of cellulose adaptation and metabolism by pure cultures was similar to the methods described in the previous section dealing with bile pigments and hestianic acid. No problem was encountered in developing a mixed culture capable of metabolizing cellulose as a sole carbon source. Difficulty was encountered in growing an adequate quantity of cells on cellulose for the Warburg studies.

TABLE X
Oxygen uptake and oxidation ratios for mixed and pure cultures

Substrate	Input COD ($\mu\text{g.}$)	Net O_2 utilized ($\mu\text{g.}$)	Net O_2 utilized
			COD input
3BCDE			
Raw waste	980	495	0.505
Bilirubin	980	125	0.128
Cyclopentane	10,400	100	0.0096
Pyrrole	6,450	0	No metabolism
Pyrazole	300	78	0.260
3AF			
Raw waste	980	500	0.510
Bilirubin	980	35	0.0357
Biliverdin	500	0	No metabolism
Pyrrole	300	10	0.030
Pyrazole	300	49	0.163
Bilirubin			
Raw waste	1,030	552	0.536
Bilirubin	1,070	117	0.109
Cyclopentane	10,400	136	0.013
Pyrrole	300	0	No metabolism
Pyrazole	300	0	No metabolism

TABLE XI
Oxygen uptake and oxidation ratios for mixed and pure cultures

Substrate	Input COD ($\mu\text{g.}$)	Net O_2 utilized ($\mu\text{g.}$)	Net O_2 utilized COD input
Pyrrole			
Cyclohexane	1,000	165	0.165
Cyclopentane	1,000	229	0.229
Pyrazole	300	0	No metabolism
Pyrrole	300	0	No metabolism
Bilirubin	980	9	No metabolism
Pyrrole - raw waste			
Pyrrole	692	60	0.0868
Bilirubin	990	165	0.167
Raw waste	103	415	4.02

A great deal of time was expended in attempting to transfer the organisms from a mixed mother culture to small sterile vessels containing mineral salts and cellulose. These attempts were not successful in that no utilization of cellulose could be demonstrated in the Warburg apparatus over a 24-hour period, although some cell proliferation did occur.

Later attempts at organism isolation involving the use of sterile waste enriched with cellulose were more successful. In summary—

1. Cellulose suspended in distilled water failed to give any growth.
2. Cellulose agar (1% agar + 5% cellulose in phosphate buffer) also gave no growth.
3. Cellulose agar (1% agar + 1% cellulose, 0.5% peptone in phosphate buffer) gave only a little growth.
4. Raw waste was diluted to 6 gm./liter. A cellulose suspension at 25 gm./liter was prepared with the usual phosphate ammonia buffer. A series of 8 tubes containing 8 ml. of a sterile mixture of the constituents in method 4 was prepared according to table XII.

The tubes were inoculated from nutrient agar slants and incubated for a total of 70 days

TABLE XII
Media employed for growth of cellulose-using organisms

Tub. No.	Raw waste* (ml.)	Cellulose* (ml.)
1	8	0
2	6	2
3	4	4
4	3	5
5	2	6
6	1	7
7	0.5	7.5
8	0	8

*In each portion

at 37°C. Good active growth was found in all tubes ranging from raw waste to 100% cellulose with the following organisms: 2FC, 2GH, 6B, 7B, 7F, and 8A.

Plate counts were made from the cultures prepared by method 4 to indicate the concentration. The 100% cellulose culture was used for the plate count, diluted in tryptose blood agar

base with dilutions of 1/1,000 and 1/10,000. Pour plates were incubated for 18 hours. Colony counts resulted in the following yields:

Organism	Organisms per milliliter
2FC	2,000
2GHI	7,848,000
6B	4,184,000
7B	381,000
7F	583,000
8A	148,000

After the completion of the work described in the previous paragraph, Warburg studies of cellulose metabolism were carried out with the bulk of the organisms harvested for Warburg study as previously described. These organisms showed a surprising amount of cellulolytic activity with little of the lag time which normally characterized cellulose utilization by activated sludge. The data concerning cellulose utilization by the pure cultures were presented in the section dealing with the metabolism of the pure cultures.

VI. DISCUSSION

Significance of observed metabolic patterns

The results of the metabolism studies permit a broader characterization of the activated sludge process than heretofore possible. Although lags and other metabolic aberrations prevented the acquisition of quantitative data, the use of raw waste and glucose as standard substrates permits comparisons which can serve in lieu of more specific quantitative information. Every effort was made to maintain the cultures, both mixed and pure, in the best condition to insure uniformity of behavior. Wherever possible, replicate tests were separated by long periods, greater than 2 weeks, to evaluate any problems introduced by mutation and reversion.

The organisms identified in the present work have, for the most part, been previously noted in activated sludge. Hence, it is assumed that the comments pertaining to activated sludge operation probably are general and apply equally to the high-solids and conventional system.

It has been frequently postulated that no one organism could possibly carry out the steps required for all the saprophytic activity seen in activated sludge. Ingram (20), as mentioned earlier, made such a statement in his review of activated sludge microbiology. The concept is derived from the assumption that a tightly controlled symbiosis exists in activated sludge; a relationship in which organism A alters substrate A' to B', then organism B alters B' to C', and so on. Hence, organism B removes waste product A', and so on—a true symbiosis.

What has been observed in the present work, however, does not support the concept of a symbiotic relationship. What is seen, instead, is a commensal relationship, in which the activity of a few macromolecule-users benefits many other organisms unable to assimilate complex substrates. The data obtained in this study permit a clearer understanding of the commensal relationship.

To provide a means of evaluating the results of the metabolism study, tables XIII and XIV have been prepared. The data presented therein have been organized in such a fashion as to provide a means of recognizing the more resistant substrates and to evaluate the effects of commensalism.

Although many substances did not appear to undergo complete metabolism when offered singly, Okey and Bogan (26) have shown that the presence of alternate substrates will assist in the complete assimilation of a new or somewhat refractory material. For this reason, oxidation ratios of 0.3 to 0.4 may well be 0.6 to 0.7 when the test substrate is augmented with additional metabolites. Hence, an oxidation ratio of 0.3 has been arbitrarily chosen to represent substantial but incomplete metabolism. The lower limit of 0.1 was used in table XV because it roughly represents the limit of reproducibility of the Warburg procedure.

The existence of commensal relationships can be noted in tables XIII, XIV, and XV by observing the extent of metabolism by mixed or bulk cultures as compared to that of the

TABLE XIII
Observed index of substrate biodegradability

Substrate	Number of tests	Examinations yielding oxidation ratios > 0.6				Isolates with oxidation ratios > 0.6 (%)
		Total	Mixed	Bulk	Pure	
Uric acid	10	5	2	NT	3	37.5
Hippuric acid	10	2	1	NT	1	12.5
Hexanoic acid	19	1	1	NT	0	0
Creatinine	17	4	2	0	2	12.5
Creatine	14	7	1	2	4	50.0
Starch	14	1	0	1	0	0
Cellulose	16	0	0	0	0	0
Phenylsulfate	13	0	0	0	0	0
Triton X-100	13	1	0	1	0	0
Stearic acid (sodium salt)	17	4	1	1	2	25.0
Glycocholate	15	6	2	5	1	12.5
Taurocholate	14	8	2	2	4	50
Albumin	14	5	3	1	1	12.5
Tripalmitin	14	0	0	0	0	0
Urochrome	12	0	0	0	9	9
Coproporphyrin	17	0	9	0	0	0
Dextrose	25	8	1	2	5	67.5
Raw waste	35	14	2	3	8	75.0

Data not corrected for nitrification.

NT = Not tested.

TABLE XIV
Observed index of substrate biodegradability

Substrate	Number of tests	Examinations yielding oxidation ratios > 0.3				Isolates with oxidation ratios > 0.3 (%)
		Total	Mixed	Bulk	Pure	
Uric acid	10	8	2	NT	3	75.0
Hippuric acid	10	6	2	NT	4	50.0
Hexanoic acid	10	4	1	NT	3	62.5
Creatinine	17	7	2	1	4	38.5
Creatine	14	11	1	4	6	62.5
Starch	14	3	1	2	0	0
Cellulose	16	2	0	1	1	12.5
Phenylsulfate	13	5	0	1	4	37.5
Triton X-100	13	5	0	2	3	37.5
Stearic acid (sodium salt)	17	12	1	3	8	75.0
Glycocholate	15	10	2	3	5	62.5
Taurocholate	14	9	2	2	5	62.5
Albumin	14	6	3	1	2	12.5
Tripalmitin	14	2	1	0	1	12.5
Urochrome	12	0	0	0	0	0
Coproporphyrin	17	2	1	1	0	0
Dextrose	25	21	3	4	14	100
Raw waste	35	23	5	3	15	100

Data not corrected for nitrification.

NT = Not tested.

TABLE XV
Observed index of substrate biodegradability

Substrate	Number of tests	Examinations yielding oxidation ratios < 0.1				Isolates with oxidation ratios < 0.1 (%)
		Total	Mixed	Bulk	Pure	
Uric acid	10	0	0	NT	0	0
Hippuric acid	10	1	0	NT	1	12.5
Hexanoic acid	10	2	0	NT	2	25.0
Creatinine	17	5	0	0	5	37.5
Creatine	14	2	0	0	2	14.3
Starch	14	9	0	0	9	87.5
Cellulose	16	5	1	0	4	37.5
Phenylsulfate	13	5	1	0	5	50.0
Triton X-100	13	6	2	0	4	50.0
Stearic acid (sodium salt)	17	0	0	0	0	0
Glycocholate	15	3	0	0	3	25.0
Taurocholate	14	2	0	0	2	12.5
Albumin	14	7	0	0	7	87.5
Tripalmitin	14	10	0	2	8	62.5
Urochrome	12	12	2	2	8	100
Coproporphyrin	17	10	1	1	8	62.5
Dextrose	25	3	1	0	2	0
Raw waste	35	0	0	0	0	0

Data not corrected for nitrification.

NT = Not tested.

isolates. For example, the interdependence between organisms is clearly evident in the metabolism of starch, glycocholate, taurocholate, and albumin, and possibly in raw waste and the detergent, Triton X-100. Also, based on the bulk culture metabolism of coproporphyrin, phenylsulfate, and cellulose, some commensalism may exist during the assimilation of these molecules by activated sludge. On the other hand, the bulk of the small molecules was metabolized by many of the isolates to a substantial extent. This conclusion is also evident from the data presented in tables XIII, XIV, and XV.

The origin and precise nature of the commensalism observed in this work could only be determined by repeated studies of varying combinations of the isolates obtained from the activated sludge. Such a study could add a great deal of information to our current fund of knowledge; however, it was clearly beyond the scope of the present work.

The extent of the metabolism of urochrome was extremely low by both the mixed and pure cultures. Because it was necessary to eliminate any volatiles from the Warburg flask, it is possible that some of the urochrome was lost which would, in effect, produce a lower oxidation ratio. It would appear that additional studies with more refined technics, both of isolation and of sample preparation, should be carried out to further define the picture concerning urochrome metabolism. This is the only instance in which it was not possible to assure a good quality substrate in the Warburg studies.

The results obtained support the concept that many organisms can totally assimilate small molecules; however, the capability to reduce the size of the large molecules such as albumin and starch was limited. What is seen, therefore, is the widespread capability to assimilate most small substances, but a limited capability to release small fragments from

larger molecules. The release of the small fragments probably benefits all the organisms in the mixed cultures. This pattern was noted with all substrates except cellulose. Cellulose assimilation was considerably more widespread than first anticipated. A curious aspect of the organisms metabolizing large molecules was their inability to assimilate many small molecules.

Since large molecules comprise most of the material, in terms of mass, contained in human waste, the factor of commensalism in the metabolism of large molecules has an important bearing on human waste treatment and the synthesis of a biologic system from pure cultures. It can be assumed that for human waste assimilation, commensalism is common. Commensalism would be of less importance in treating individual small molecules which are present in many kinds of industrial wastes.

It is seen, therefore, that in developing a microbial system from pure cultures to substitute for an activated sludge system, many organisms may suffice, but only two basic properties are required—large-molecule and small-molecule metabolic activity.

The most efficient system metabolizing human waste would be one employing the large-molecule users in a system without competition from other organisms. Such a system would allow a maximum rate of proliferation of large-molecule users, and could conceivably reduce the time required for overall waste assimilation. It would then be possible to follow such a system with a reactor designed for the cleanup of small molecules. It appears obvious that in the normal competitive environment of a waste treatment system receiving unsterilized waste that the required holdup time will be extended by the limited rate of growth of the large-molecule users.

Nitrification in activated sludge

The ability to produce nitrate or nitrite or act on nitrate and urea was found to be widespread in the mixed cultures. The oxidation of reduced nitrogen as an energy source, as seen

in the Warburg procedure, is characterized by an oxygen use greatly in excess of that to be expected in theory. This occurrence prevents a reliable determination of the extent of substrate oxidation. There appears to be no way, however, that the oxidation of nitrogen can be prevented in those instances in which excess nitrogen is present in the substrate.

It has been assumed that the pure culture work would not be hampered by extensive nitrification, as it is commonly held that the oxidation of nitrogen is carried out primarily by the autotrophic nitrifiers, *Nitrosomonas* and *Nitrobacter* (16, 21). In addition, these organisms are not thought to be heterotrophs. There is some evidence that some of the isolates examined, while being saprophytic, may also utilize the oxidation of nitrogen as a source of energy. It appears certain that the 5-bulk cultures during the metabolism of creatine, glycocholate, taurocholate, and dextrose were given the impetus to carry out some oxidation steps beyond substrate oxidation not carried out by the control cultures. The tests for nitrite and nitrate showed that, in some instances, compounds were formed during incubation which gave positive reactions for one or both of these materials.

It is tentatively suggested, then, that some of the commonly encountered saprophytes can, under some conditions, use reduced nitrogen as an energy source. If proved to be the case, such a finding might explain why an activated sludge system after long periods in a low dissolved-oxygen environment and heavy organic load (which would provide little opportunity for the growth of purely autotrophic organisms) will suddenly produce large quantities of oxidized nitrogen. Such an observation was made by the Boeing Company during the "Mesa" manned chamber run (29).

As a practical matter, it would appear to be desirable to suppress the oxidation of ammonia in a remote environment. First, the oxidation of nitrogen yields energy which permits a higher synthesis rate. Second, ammonia, because of its relatively greater adsorbability, gaseous nature, and ease of detection, appears

to be an easier contaminant to handle than nitrite or nitrate.

In the Boeing "Mesa" manned chamber run, high rates of conversion of ammonia to nitrite were observed when dissolved oxygen levels were maintained above 0.5 mg./liter. Therefore, control of dissolved oxygen levels below that point may prevent ammonia oxidation. It is also axiomatic that, in a reactor, thorough scrubbing of ammonia will reduce the available nitrogen to minimum levels. Another interesting possibility is the removal of ammonia as nitrogen. The latter control mechanism is possible because of the peculiar biochemistry of the nitrate and nitrite users, which will, under conditions of partial anaerobiosis, convert the bulk of the oxidized nitrogen to nitrogen gas. Hence, it appears theoretically possible to convert the plant effluent ammonia to nitrate or nitrite and then, under conditions of low- or zero-dissolved oxygen, convert the nitrate or nitrite to nitrogen gas. Johnson and Schroeppel (21) have studied such a flow sheet and found it to function as described.

Bilirubin and pyrrole metabolism

Despite repeated efforts, substantial metabolism of bilirubin or pyrrole could not be demonstrated. Some of the organisms studied, however, were able to assimilate coproporphyrin to a substantial extent. These two molecules both contain a tetrapyrrole group. Bilirubin is a linear tetrapyrrole and coproporphyrin contains the four pyrrole rings in the basic heme configuration.

Aerobic organisms contain enzymes of the terminal respiration sequence which possess the heme configuration. It is natural to assume that some internal mechanism exists for the conservation of the nitrogen contained in the pyrrole and the utilization of the molecule itself. The results of the present study show that the tetrapyrroles are at least moderately refractory.

Although it may be only coincidental, the behavior of sodium bilirubinate and hestianic

acid was very similar. According to Emanuel (11), hestianic acid contains nitrogen and is also aromatic, indicating that at least part of the substances contained in hestianic acid may also be derivatives of bilirubin.

The comparative ease of metabolism of the 4-, 5-, and 6-membered non-nitrogenous cyclic molecules indicated that the molecular characteristic creating the behavior was the heteronitrogen in the ring. The most profound effect that the heteronitrogen has on the molecule is to change its polar and electronic characteristics. There is some evidence which supports the concept that alterations in the foregoing characteristics may affect or reduce the rate of transport or catabolic attack (26). The findings here would appear to support this concept, particularly the portion dealing with transport, as the assumption is made that some intracellular pyrroles exist in most aerobic microbiota.

It would appear axiomatic that some mechanism must exist in nature for destroying the linear tetrapyrroles. It is also necessary to note that other types of mechanisms, purely chemical (i.e., photocatalytic), or the involvement of a highly specific saprophyte not observed here, may be responsible for the ultimate destruction of such compounds.

Adsorption of substrates by pure cultures

Ingram (20) postulated that the so-called zoogloal material or slimes excreted by many of the bacteria commonly found in activated sludge may be responsible for the rapid non-oxidative uptake of certain substrates, frequently termed biologic adsorption. The slimes appear to be excreted by organisms growing beyond the logarithmic growth phase and, therefore, would not be present in the dispersed growth obtained for study in this work.

Instead of a rapid non-oxidative uptake of substantial quantities of substrates as has been previously reported for clarified raw waste and albumin, in the present study slight to negligible amounts of substrates were removed rapidly followed by a linear (non-concentration-dependent) removal pattern.

This type of assimilation pattern is similar to that noted in earlier studies (6) for small substrate molecules. These findings would tend to support the concept that the zoogloal slime is responsible for the bulk of substrate adsorption.

The zoogloal slimes appear to result from the way the conventional activated sludge systems are operated. As the sludge mass per unit of utilizable substrate is increased, the system tends toward steady state. It is at, or near, the steady-state situation that the sludge becomes "activated" and a substantial quantity of slimes is produced. This occurrence may be a direct result of the increased competition for food. During periods of dispersed (nonfloculating) growth, where food is in excess, the slimes do not appear to form to any substantial degree. Therefore, the phenomenon of adsorption may be said to be a direct result of the mode of system operation.

Comments on the characterization of the activated sludge process

In the present work, organisms contained in high-solids activated sludges have been isolated and tested. In each instance the organisms have previously been isolated from biologic waste treatment systems, as is evidenced by Ingram's thorough review; however, the metabolic characteristics observed here of the isolates and the combinations thereof permit some significant generalizations about the process.

It appears certain that a mixed culture of at least three organisms will be required to reproduce the activity of an activated sludge facility. Although time did not permit such an appraisal in the laboratory, an analysis of the oxidation ratio data indicates that organisms capable of assimilating the complex molecules, and one other capable of substantial small-molecule activity could conceivably suffice, although a combination of three or more organisms might complete oxidation faster.

It also appears certain that nitrification will occur unless strict control of the dissolved

oxygen level in the reactor is practiced. The production of nitrite or nitrate requires oxygen for a use not directly associated with waste stabilization and produces a substance, oxidized nitrogen, deleterious to man. The latter is particularly true in remote environments. For the foregoing reasons, nitrification appears undesirable and control should be exercised to prevent it.

The adsorption phase of substrate uptake appeared to be absent or negligible in the studies of pure cultures. The adsorption phase has been shown to be characterized by a rapid nonoxidative uptake of substrate from the medium. This uptake has been shown to closely follow a first-order pattern based on studies by Chapman et al. (6). It has been postulated that the bacterial slimes associated with activated sludge are responsible for the process of substrate adsorption. This postulate is supported, but not proved, by the findings of the present study. These findings, if substantiated by further work, imply that dispersed organisms, while capable of oxidizing the components of waste, would not rapidly remove the adsorbable components. This would probably result in a lower overall efficiency in a continuous flow-through facility.

Based on the data obtained in this and previous studies, figure 11 has been prepared. Figure 11 represents a summary of the information obtained to date on the mechanism of the activated sludge process as listed in the following paragraphs:

1. Most large molecules—i.e., starch, albumin, and the constituents of human waste—appear to be rapidly and nonoxidatively adsorbed onto the biologic matrix.
2. Most small molecules, such as phenol, individual amino acids, and hippuric acid, appear to be metabolized as rapidly as removed from the medium.
3. Most small molecules appear to be utilized by organisms incapable of large-molecule assimilation.

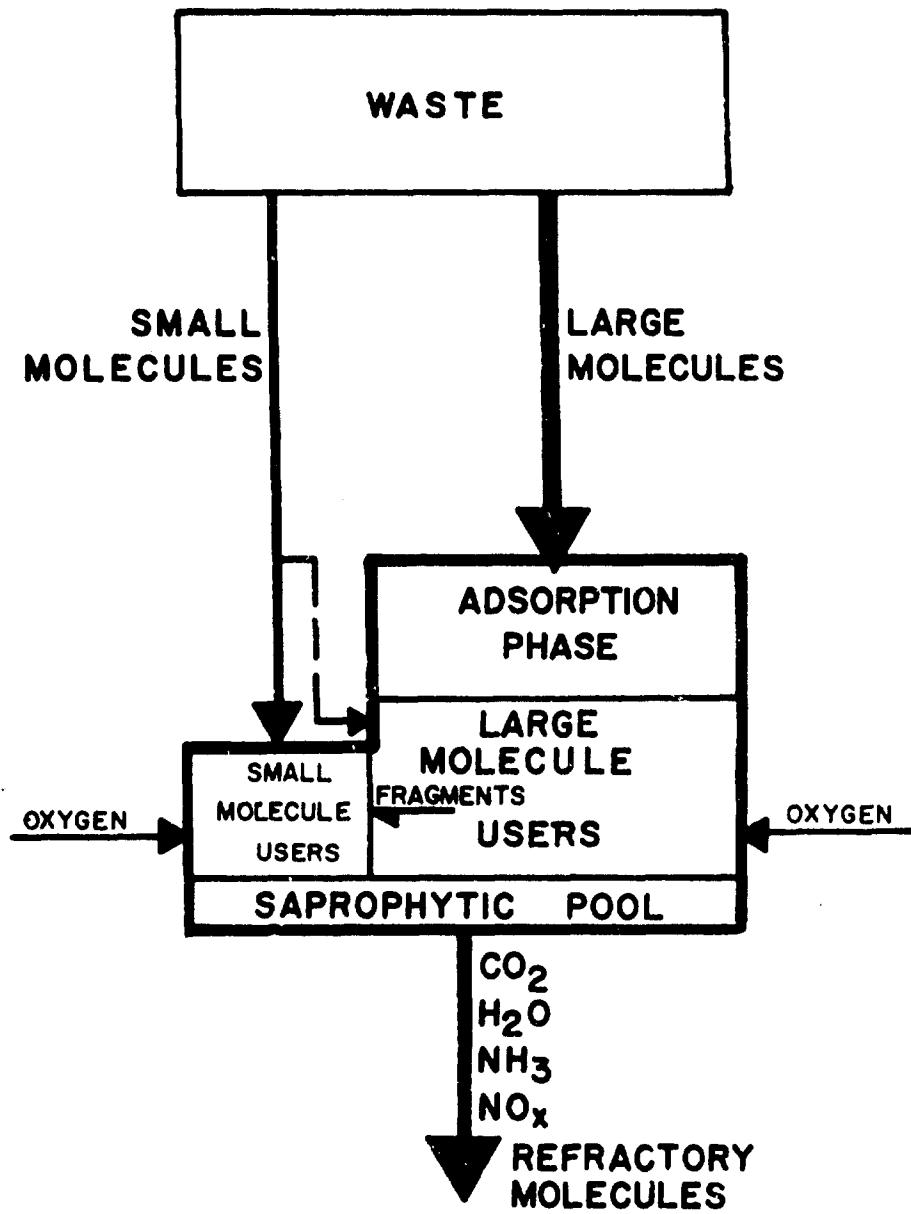


FIGURE 11
Characterization of the activated sludge process.

4. The organisms assimilating large molecules metabolized the small molecules sparingly.

5. Refractory molecules are present in human waste and appear in some combination in the effluent from an activated sludge facility.

6. The gaseous effluent will contain CO₂, NH₃, and H₂O, and, depending upon the level of aeration, some oxides of nitrogen.

7. The oxidation of most small molecules will be complete within minutes or hours. The larger molecules and those small molecules tending to be refractory will require additional time for stabilization. It is probable that the 3 or 4 days apparently required for good stabilization in a high-solids activated sludge is due to the extension of time required to stabilize the complex or large molecules.

VII. RECOMMENDATIONS FOR FURTHER STUDY

Pure culture work

Continued key-out studies are necessary for more specific identification of the isolated organisms. This work should include rechecking some of the results obtained in the present program to eliminate the possibility of reversion from a mutant strain, or the possibility of a strain becoming attenuated through repeated passaging. Animal inoculation or regrowth through raw waste is also indicated. The animal inoculation would be carried out to develop any lost chromogenesis or fluorescent capabilities of these organisms. Serologic classification of some of these organisms is indicated, but comparative study to type cultures would be the best method of final identification.

Indications that the saprophytes may, under some circumstances, use the oxidation of nitrogen as an energy source should lead to a

thorough study of the phenomenon in activated sludge. The control of synthesis to maintain a minimum quantity of cellular material in the reactor is desirable and perhaps necessary for remote environment application.

Further studies on bilirubin, pyrrole, and coproporphyrin metabolism are indicated as well as additional studies on urochrome. It is suggested that attempts be made to maintain the organisms in pure culture under macroculturing conditions and that substrate depletion studies be carried out over extended periods—that is, weeks or months.

Mixed culture studies

Additional studies should be directed toward control of synthesis in mixed cultures. The work should be carried out with two objectives: first, to minimize the utilization of reduced nitrogen as an energy source; and second, to reduce the normal rate of synthesis by the saprophytes during heterotrophic metabolism. There are a number of compounds capable of reducing the rate of synthesis. The use of these compounds should be studied.

Attention should also be directed to a selective elimination of inactive and dead material contained in the biologic matrix. It has been previously suggested that it may be possible to improve the operation of an activated sludge system per unit mass of material by eliminating the so-called "dead weight" material. Such problems as foam, gas transfer, and movement of mixed liquor may be reduced substantially by such a development.

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Unclassified

Security Classification

DOCUMENT CONTROL DATA - R & D

1. SECURITY CLASSIFICATION OF THIS REPORT		(Security classification of title, body of abstract and indexing annotation must be entered when the overall report is classified)	
1a. ORIGINATING ACTIVITY (Corporate author) Scientia Research Laboratories, Inc. Seattle, Washington		1b. REPORT SECURITY CLASSIFICATION Unclassified	
		1c. GROUP	
2. REPORT TITLE MICROBIOLOGIC STUDIES OF THE ACTIVATED SLUDGE PROCESS FOR THE RECYCLING OF HUMAN WASTES			
3. DESCRIPTIVE NOTES (Type of report and inclusive dates) Final report June 1963 - Dec. 1964			
4. AUTHOR(S) (First name, middle initial, last name) D. Duane Chapman Robert W. Okey Fredric T. Santler			
5. REPORT DATE September 1968		6. TOTAL NO. OF PAGES 41	7. NO. OF REFS 44
8. CONTRACT OR GRANT NO. AF 41(600)-1974		9. ORIGINATOR'S REPORT NUMBER (RIS) SAM-TR-68-77	
10. PROJECT NO. Task No. 793001		11. OTHER REPORT NO(S) (Any other numbers that may be assigned to this report) d.	
12. DISTRIBUTION STATEMENT This document has been approved for public release and sale; its distribution is unlimited.			
13. SUPPLEMENTARY NOTES		14. SPONSORING MILITARY ACTIVITY USAF School of Aerospace Medicine Aerospace Medical Division (AFSC) Brooks Air Force Base, Texas	
15. ABSTRACT <p>A study was carried out to evaluate the assimilability of common urinary and fecal constituents by the active saprophytes of high-solids activated sludge. The saprophytes were obtained in pure culture from mixed cultures grown on undiluted human waste. Conventional isolation and determinative procedures were employed. The isolated organisms were found to be primarily species of <u>Alcaligenes</u>, <u>Pseudomonas</u>, and <u>Achromobacter</u>, all of which have been previously identified in activated sludge. It was observed that most organisms could assimilate a substantial quantity of the small molecules, such as uric and hippuric acids, contained in human waste. The ability to handle complex polymeric substrates, however, such as starch, albumin, and cellulose, was found to be limited. Bilirubin, coproporphyrin, and tribalmitin were found to be refractory. The authors attribute the saprophytic activity in activated sludge to a commensal rather than to a symbiotic relationship among the organisms. Commensalism has an important bearing on synthesis of a biologic system from pure cultures. It appears that at least three organisms will be required to reproduce the activity of an activated sludge facility. Further study is recommended in these areas: more specific identification of isolated organisms; use by saprophytes of oxidation of nitrogen as an energy source; bilirubin, pyrrole, and coproporphyrin metabolism; control of synthesis in mixed cultures; and selective elimination of inactive material in the biologic matrix.</p>			

~~Unclassified~~

Security Classification

14 KEY WORDS	LINK A		LINK B		LINK C	
	ROLE	WT	ROLE	WT	ROLE	WT
Bioastronautics Waste management Biologic waste treatment Activated sludge Commensalism Biodegradation Bacteria in activated sludge Metabolic patterns of microorganisms						

~~Unclassified~~

Security Classification